Universidade de Lisboa

Faculdade de Medicina Dentária

Effects of Salivary Acetylcholinesterase on the Cytotoxicity of Acrylic Reline Resins

Miguel Constantino Mendes de Oliveira

Mestrado Integrado

2013

Universidade de Lisboa

Faculdade de Medicina Dentária

Effects of Salivary Acetylcholinesterase on the Cytotoxicity of Acrylic Reline Resins

Miguel Constantino Mendes de Oliveira

Dissertação orientada pela Professora Doutora Cristina Bettencourt Neves

Mestrado Integrado

2013

Este trabalho é dedicado aos meus pais Vítor e Beatriz,
ao meu irmão Pedro,
e à Rita,
o amor da minha vida.

Table of Contents

Agradecimentos…….. v

Resumo……..…… vii

Abstract…….. xi

1. Introduction……... 1

2. Materials and Methods…….. 5

2.1. Chemicals…….. 5

2.2. Cell Culture……... 5

2.3. Preparation of the test specimens…….. 7

2.4. Preparation of the test eluates……... 9

2.5. Cytotoxicity assay……. 10

2.5.1. MTT assay……... 10

2.5.2. LDH assay…… 11

2.6. Preparation of the test compounds and acrylic reline resin liquids……... 13

2.7. Statistical Analysis…… 13

3. Results……... 14

3.1. Cytotoxicity of the eluates…… 14

3.1.1. MTT assay……... 14

3.1.2. LDH assay……..... 15

3.2. Cytotoxicity of the test compounds solutions……..……. 16

3.3.Cytotoxicity of the acrylic reline resins liquids……. 17

4. Discussion……. 19

5. Conclusions……... 27

6. References……. 29

7. Appendix……... 39

7.1. List of Figures and Tables……..…….. 39

7.2. List of Abbreviations…… 41

7.3. Experimental data…….. 43

7.3.1. Cytotoxicity of the eluates……. 43

7.3.2. Cytotoxicity of the test compounds solutions……..…….. 57

7.3.3. Cytotoxicity of the acrylic reline resin liquids……... 62

7.4. IC50 determination tables……..... 66

Agradecimentos

Gostaria de expressar a minha gratidão à Professora Doutora Cristina Neves, Professora Auxiliar da Unidade Curricular de Clínica de Reabilitação Oral I e II e orientadora desta tese, pela oportunidade de integrar a minha tese nesta área tão aliciante que resulta da fusão da Prostodontia com as Ciências Básicas, pelas quais tenho um especial interesse. A sua disponibilidade na orientação deste trabalho, bem como o incentivo à autonomia e ao espírito crítico, permitiram-me evoluir enquanto estudante de Medicina Dentária e enquanto futuro Médico Dentista.

Ao Professor Doutor António Mata, Professor Associado com agregação e Regente das Unidades Curriculares de Imunologia e Biologia Oral I e II da FMDUL e Investigador Principal do GIBBO, o meu profundo agradecimento pelos já 4 anos de orientação, durante os quais pude comprovar a constante capacidade de trabalho e de motivação, rigor científico e amizade para com os colaboradores, tornando este grupo, cada vez mais, uma referência no panorama científico na área da Medicina Dentária.

Ao Professor Doutor Duarte Marques, Professor Convidado e Regente das Unidades Curriculares de Anatomia I e II, Anatomia de Cabeça e Pescoço e Fisiologia da FMDUL, um sentido obrigado pelos conselhos e ensinamentos ao longo da minha colaboração com o GIBBO. A par do supracitado Professor Doutor António Mata, considero ser uma das mentes mais brilhantes da Medicina Dentária portuguesa.

Ao Doutor João Silveira, pelo companheirismo, tutoria e confiança transmitidas. De vastos conhecimentos científicos, partilha-os sem contrapartidas. Não conseguirei agradecer o suficiente o tempo disponibilizado na minha aprendizagem.

À Doutora Joana Marques, com quem tive a oportunidade de colaborar na elaboração de alguns projectos de investigação, devo-lhe um forte agradecimento pela sua influência no meu crescimento académico. Desejo-lhe o maior sucesso na finalização da tese de Doutoramento.

Ao Doutor Nuno Guilherme e João Amaral pela amizade com que me presentearam ao longo destes anos. Recordo com saudades as tardes de Agosto no IPR ou no laboratório, os congressos do IADR em Barcelona ou San Diego. Obrigado.

À Professora Doutora Manuela Lopes, Regente das Unidades Curriculares de Biologia do Desenvolvimento, Biologia Celular e Molecular e Genética Molecular

Humana da Faculdade de Medicina Dentária da Universidade de Lisboa, pela minha introdução no mundo da investigação. Foi a alegria contagiante com que falava da investigação nas suas aulas que fez despertar esta vontade de querer saber mais, e tentar produzir ciência. Um profundo obrigado.

Aos restantes colaboradores do GIBBO, quero agradecer as longas horas de trabalho que partilhámos e que as transformaram em boas memórias.

À Professora Doutora Ana Francisca Bettencourt, Professora Auxiliar de Biomateriais e Química-Física da Faculdade de Farmácia da Universidade de Lisboa, gostaria de expressar a minha gratidão pela óptima recepção e integração no grupo de investigação do "Chemical Biology and Toxicology Group" do Imed.UL da Faculdade de Farmácia da Universidade de Lisboa, e pelo acompanhamento na realização desta tese.

À equipa do grupo de investigação do "Chemical Biology and Toxicology Group" do Imed.UL da Faculdade de Farmácia da Universidade de Lisboa, por me ter recebido com toda a disponibilidade e simpatia. Em especial à Doutora Joana Miranda, pelo seu apoio, perspicácia e rigor metodológico. À Elysse Filipe, Patrícia Guerreiro, João Costa, Pedro Pinheiro, Madalena Cipriano e Alexandra Silva, pela disponibilidade, interesse e experiência demonstradas na valiosa ajuda nos estudos de viabilidade celular. Ao Professor Doutor Nuno Oliveira, e Professoras Doutoras Fátima Cabral e Judite Costa pela simpatia e apoio demonstrados.

Por fim, mas não menos importante, um agradecimento muito especial ao meu grande Amigo, e dupla, João Godinho, com quem partilhei, nestes últimos 3 anos e meio, não só pacientes, trabalhos, a paixão pela ciência, mas também muitas tardes, noites e fins-de-semana, todos, sem excepção, de muita alegria e repletos de boas memórias. Também os seus pais e irmão, Fernando, Matilde e António, merecem a minha gratidão. Durante estes anos incluíram-me e trataram-me como membro da família. Obrigado por tudo.

À minha família, as palavras que poderei proferir nunca farão jus à importância que cada um, individualmente, teve na minha formação. Particularmente aos meus pais, que me deram a liberdade e suporte que necessitei para me tornar na pessoa que sou hoje. Ao meu irmão, pelo contínuo apoio, agradeço do fundo do coração.

A ti Rita, obrigado por me fazeres feliz.

Resumo

As alterações fisiológicas resultantes da reabsorção progressiva do rebordo alveolar diminuem a íntima adaptação das bases das próteses removíveis, resultando na perda de retenção e conforto, com consequente lesão da mucosa e limitação da função mastigatória. As resinas de rebasamento duro autopolimerizáveis têm sido utilizadas para readaptação das próteses ao rebordo alveolar, providenciando melhor retenção e estabilidade às mesmas. Os rebasamentos são uma opção terapêutica útil e relativamente barata, quando comparados com a realização de novas próteses.

Os procedimentos de rebasamento podem ser classificados como directos ou indirectos, conforme são realizados directamente na cavidade oral ou por intermédio de procedimentos laboratoriais, respetivamente.

As resinas acrílicas pertencem à maior classe dos biomateriais – os polímeros. Um polímero resulta de uma cadeia longa de pequenas unidades repetidas, designadas de monómeros. Estas unidades poliméricas resultam de um processo de polimerização adicional de radicais livres onde um iniciador, geralmente o peróxido de benzoílo, quebra a ligação dupla do monómero, ficando exposto um local de ligação para o contínuo crescimento. Desta reação de polimeração resultam monómeros residuais, que permanecem não polimerizados.

Estes materiais são compostos por um pó, que contém o iniciador da reacção e um líquido, que pode conter monómeros como isobutil-metacrilato (IBMA), butil-metacrilato (BMA), 2-hidroxietil-metacrilato (HEMA) ou 1,6-hexanediol-dimetacrilato (1,6-HDMA) com ou sem agente de ligação.

Os materiais das bases das próteses removíveis têm sido associados a reacções alérgicas, incluindo eritema, erosão da mucosa oral e síndrome da boca ardente. Entre os profissionais de saúde oral estão também descritas reações alérgicas, pela manipulação destes materiais. Estes efeitos adversos têm sido atribuídos a substâncias lixiviadas destes materiais, especialmente, os monómeros residuais não polimerizados.

Este processo de lixiviação ocorre por penetração da água na matriz dos polímeros, com consequente expansão das cadeias poliméricas, permitindo a difusão dos monómeros residuais para a saliva e mucosa oral adjacente à base das próteses.

A quantidade de monómero lixiviado está dependente de factores, como o tipo de resina, a composição da mistura, o tipo de reacção de polimerização, a natureza do iniciador, a duração do ciclo de polimerização, a espessura da resina e o método de polimento. No entanto, o grau de conversão de um determinado monómero é o factor mais importante sobre as propriedades mecânicas do polímero e a quantidade de monómero lixiviado.

Diversos estudos revelam que os polímeros estão sujeitos a inúmeros processos de biodegradação na cavidade oral. A biodegradação é o processo de desgaste de um material mediado por organismos vivos, levando a alterações das propriedades físicas. Na cavidade oral, a degradação é um processo complexo no qual está incluída a dissolução dos materiais pela saliva. Esta dissolução ocorre devido à hidrólise química e enzimática dos grupos éster das cadeias poliméricas dos metacrilatos. Estes grupos éster são particularmente susceptíveis à hidrólise por esterases salivares, incluindo a acetilcolinesterase. Em contraste com os compósitos, existe muito pouca informação sobre a biodegradação de resinas acrílicas na presença de esterases.

O objectivo deste trabalho foi avaliar o efeito da enzima salivar acetilcolinesterase na biodegradação de três resinas acrílicas de rebasamento (Kooliner, Ufi Gel Hard e Probase Cold) e o seu efeito biológico em culturas primárias de fibroblastos da derme. Para tal, foram utilizados dois testes funcionais colorimétricos, o MTT e o LDH, para avaliação da actividade das desidrogenases mitocôndriais e a actividade das desidrogenases lácticas, respetivamente. Foi avaliada, também, a citotoxicidade dos líquidos das resinas acrílicas de rebasamento, através do IC50, e dos respetivos compostos puros (monómeros) IBMA (Kooliner), MMA (Probase Cold), HDMA (Ufi Gel Hard) e do produto de degração MA.

Para avaliação da citotoxicidade dos monómeros lixiviados (extractos) das resinas acrílicas de rebasamento estudadas, foram preparados seis espécimes de cada material em moldes de aço pré-formados com um diâmetro de 50±0.1 mm e 2±0.01 mm de espessura, representando a área de superfície de uma prótese removível total e cumprindo os critérios recomendados pela ISO (International Organization for Standardization) para a avaliação biológica de biomateriais.

Os espécimes foram incubados em 5mL de meio de cultura (grupo controlo) ou em 5mL de meio de cultura com 5U/mL de AChE (grupo experimental), durante 72

horas a 37ºC. As culturas primárias de fibroblastos humanos foram, então, expostas a várias concentrações dos extractos.

A viabilidade celular não foi afectada em todas as concentrações do extracto dos espécimes controlo de Probase Cold, enquanto o Kooliner e o Ufi Gel Hard reduziram a viabilidade em 90 e 51%, respectivamente. Quando submetidos ao tratamento com AChE, comparando com o grupo controlo, os espécimes Probase Cold permaneceram não citotóxicos e a viabilidade celular dos espécimes Kooliner e Ufi Gel Hard aumentou, com diferenças estatisticamente significativas. O ensaio do LDH revelou-se pouco sensível na avaliação da citotoxicidade dos biomateriais testados.

Quanto à citotoxicidade dos compostos puros, com base nos valores de IC50, o 1,6-HDMA revelou ser o mais citotóxico, seguido do IBMA e do MA. O MMA não provocou alterações na viabilidade celular em nenhuma das concentrações utilizadas.

No que diz respeito à citotoxicidade dos líquidos, o Ufi Gel Hard mostrou ser o mais citotóxico, seguido do Kooliner e do Probase Cold.

O facto dos extractos controlo do Kooliner se terem revelado mais citotóxicos do que os do Ufi Gel Hard, ao contrário do obtido na análise da citotoxicidade dos compostos puros e dos líquidos, pode ser explicado pela maior quantidade de monómero residual no primeiro. O aumento do monómero residual pode ser explicado pelo rácio pó/liquido inferior do Kooliner, de acordo com Kedjurane e col. 1999.

O aumento da viabilidade celular nos espécimes Kooliner, submetidos ao tratamento com AChE, poderá ser explicado pela redução da concentração de IBMA. Estudos anteriores mostraram a existência de uma reacção de degradação enzimática do referido monómero, com consequente formação de MA. Na análise da citotoxicidade dos compostos puros, MA revelou ser menos citotóxico que IBMA. Já o aumento da viabilidade celular do Ufi Gel Hard não pode ser explicado da mesma forma, uma vez que este revelou ser resistente à reacção enzimática, de acordo com Chaves e col. em 2010 e Neves em 2012.

Face aos resultados obtidos na análise da citotoxicidade dos compostos puros, a presença dos monómeros 1,6-HDMA e IBMA explica os efeitos citotóxicos observados dos líquidos do Ufi Gel Hard e do Kooliner, respectivamente. Já o MMA não consegue explicar os efeitos do líquido do Probase Cold na viabilidade celular. A citotoxicidade

do líquido do Probase Cold pode dever-se à hidrólise enzimática do MMA em MA, que, segundo os nossos resultados, é mais citotóxico.

Dentro das limitações deste estudo, podemos concluir que a resina de rebasamento Probase Cold demonstra não possuir efeito citotóxico nos fibroblastos humanos. As resinas Kooliner e Ufi Gel Hard demonstraram um comportamento citotóxico severo e moderado, respectivamente. O tratamento com aceticolinesterase não altera o efeito não-citotoxico do Probase Cold e aumenta ligeiramente a viabilidade das duas resinas de rebasamento directo.

Nas concentrações disponíveis na cavidade oral, neste estudo os monómeros puros não demonstraram efeitos citotóxicos quando expostos a fibroblastos primários humanos. Estes estão ordenados, por ordem decrescente de citotoxicidade, em HDMA>IBMA>MA.

No futuro, seria importante avaliar quais as concentrações de genotoxicidade destes materiais, face à possibilidade de terem efeito directo sobre o DNA e inviabilizarem desta forma a actividade celular, sem causar morte. A realização de uma análise morfológica por citometria de fluxo seria também pertinente, com o objectivo de avaliar os efeitos citopatogénicos das resinas acrílicas de rebasamento, uma vez que a morte celular por necrose possui um significado biológico diferente da apoptose.

Palavras-chave: Resinas Acrílicas, Biocompatilibidade, Cultura Celular, Enzimas e Fibroblastos.

Abstract

The use of autopolymerizing acrylic reline resins has recently gained popularity to readapt dentures to the continuous reabsorbed underlying tissues. However, these materials have been associated with higher levels of toxicity *in vitro*, and chemical irritation and allergic reactions *in vivo*. These biomaterials are subject of degradation in the oral cavity, in which enzymatic activity oh hydrolases plays an important role, particularly the acethylcholinesterase enzyme.

The main objective of this study is to assess the effect of a salivary esterase on the cytotoxicity of three acrylic reline resins, two directs, Kooliner and Ufi Gel Hard, and one indirect, Probase Cold, using two colorimetric functional assays, MTT and LDH. This work will try to assess the cytotoxicity of the monomers using pure compounds of IBMA, HDMA, MMA and their common by-product, MA, taking into account the IC50. The IC50 of the acrylic reline resin liquids was also studied.

In this study, the exposure of fibroblasts to direct reline resins, Kooliner and Ufi Gel Hard, eluates resulted in a significant suppression of fibroblastic function, characterized by supressed mitochondrial activity. On the contrary, Probase Cold eluate didin't exhibit cytotoxic activity. The LDH assay was found to be less sensitive that the MTT assay when assessing the cytotoxic effect of the evaluated materials.

Cells incubated with eluates treated with acethylcholinesterase changed their response to eluates from direct reline resins. The experimental specimens revealed an increase of cell viability. The non-cytotoxic effect of Probase Cold didn't change.

No cytotoxic effects were observed with the monomers, at the concentrations found to be leached in the oral cavity, when exposed to human primary fibroblasts. Considering the IC50 of the residual monomers, the cytotoxicity decreased in order of HDMA>IBMA>MA. MMA showed no biologic effect at the concentrations used.

Keywords: Acrylics, Biocompatibility, Cell Culture, Enzymes and Fibroblasts.

1. Introduction

At the present, the population age distribution in the developed countries is undergoing progressive demographic aging (Cimpan et al., 2000). The increase of life expectancy leads us to predict a wearing of complete dentures of 61 million in 2020, in the United States of America alone, compared to 53.8 million in 1991 (Douglas et al., 2002).

Denture wearers' changes in bone and soft tissue due to physiologic progression of residual ridge resorption gradually diminishes the accuracy of the denture base adaptation, resulting in loss of retention and comfort and consequent mucosal lesions (Budtz-Jorgensen, 1999) as well as impaired masticatory function (Léon et al., 2008). Autopolymerizing hard reline resins have been used to improve the adaptation of loose denture bases, providing better retention and stability for complete removable prostheses (Bohnenkamp, 1996; Aydin et al., 1999; Urban et al., 2009). Relining procedures allow a time-saving, convenient and relatively inexpensive prosthodontic treatment when compared to the cost and time-consuming new dentures (Bohnenkamp et al., 1996; Rawls, 2003; Sato et al., 2007).

The relining procedures can be classified as direct as they are performed directly in the mouth, or indirect - laboratory-processed relines (Bohnenkamp, 1996; Cucci et al., 1999).

Acrylic resins belong to the largest class of biomaterials, namely polymers. A polymer is a large molecule characterized by a long-chain bonded together by smaller repeating units called monomers – acrylic acid esters (Autian, 1975; Cooper et al., 2004; Kournetas, 2005). These polymeric units are the result of a free radical additional polymerization reaction where the initiator (usually benzoyl peroxide) opens the double bond of the monomer presenting another initiation site on the opposite side of the monomer bond for continuing growth (Lee et al., 2002; Cooper et al., 2004). From the polymerization reaction results residual monomers which remain uncured.

The powder composition of the chemically activated reline resins is based on polymethyl methacrylate (PMMA) (Celebi et al., 2008) or polyethyl methacrylate (PEMA) along with the initiator, whereas, the liquid composition varies among materials and can contain isobutyl methacrylate (IBMA), butyl methacrylate (BMA), 2-

hydroxyethyl methacrylate (HEMA) or 1,6-hexanediol dimetacrylate (1,6-HDMA) (Urban et al., 2007) with or without a cross-linking agent (Azevedo et al., 2005).

Chemical activation is accomplished through the addition of a tertiary amine activator such as dimethyl-*para*-toluidine, to the monomer, which upon mixing causes decomposition of the initiator (benzoyl peroxide), releasing free radicals to initiate the polymerization (Tandon et al., 2010). Chemical activators were introduced in 1947 in order to induce denture base polymerization at room temperature. The advantage was the great dimensional accuracy due to reduced polymerization shrinkage. Nevertheless, the great amounts of unreacted monomer in the denture base, due to incomplete polymerization, causes a decreased transverse strength and is a potential tissue irritant (Tandon et al., 2010).

In fact, denture base materials have been reported to cause local chemical irritation and allergic reactions among patients (Bohnenkamp, 1996; Huang et al., 2001; Gonçalves et al., 2006; Chaves et al., 2012;) including erythema, erosion of oral mucosa and burning sensation on the mucosa (Jorge et al., 2003; Gonçalves et al., 2008; Chaves et al., 2012), and even in dental personnel, as they manipulate those materials (Leggat et al., 2003; Aalto-Korte et al., 2007). The adverse reactions caused by denture base polymers have been attributed to substances leaching from these materials, especially unreacted residual monomers (RM), which remained in the polimerized resin net (Gonçalves et al., 2006; Celebi et al., 2008; Golbidi et al., 2009). This cytotoxicity of the resin components released have been shown to be related to lipophilicity and the mechanism of the action of the esters are believed to be membrane-mediated and relatively nonspecific (Yoshii, 1997).

The diffusion occurs as the water penetrates the matrix and expands the opening between polymer chains allowing unreacted and leachable monomers to diffuse out (Chaves et al., 2012), namely into saliva (Ebadian et al., 2008; Bural et al., 2011; Ebrahimi Saravi et al., 2012) and oral mucosa adjacent to the denture base (Bural et al., 2011).

The amount of released monomer is believed to depend on factors such as the type of resin, monomer mixture composition, polymerization reaction, the nature of the initiator system, the length of the polymerization cycle, the thickness of the resin (Geursten, 1998; Kournetas, 2005; Bayraktar et al., 2006; Ebrahimi Saravi et al., 2012) and the polishing method (Gonçalves et al., 2008). For a given monomer the degree of

conversion is an important factor (Azzarri et al., 2003) because it influences the mechanical properties of the polymer and the amount of free monomer that can be eluted from the polymer. But, although optimal mechanical properties have been achieved with high degrees of conversion, it must be considered that excessive cross-linking can lead to clinically unfavourable conditions, such as polymerization contraction (Kournetas, 2005).

Studies with infrared spectroscopy have indicated percentages of unreacted methacrylate groups from 25 to 60 %. Nevertheless, most of the unreacted carbon-carbon double bonds belong to molecules, which have reacted at one end and are thus bound to the polymer chain and are not free to elute. Still, the polymer matrix also contains a small proportion of RM. Unreacted pendant vinyl monomers, which can be hydrolysed from the resin matrix, represents a relatively labile chemical group which can define the toxicity of resin monomers (Kournetas, 2005). It has been demonstrated that autopolymerizing resins present lower degree of monomer conversion when compared with thermo-activated resins (Kedjarune, 1999; Lee et al., 2002; Takahashi et al., 2009).

Albeit the generally reliable intended lifetimes of polymeric devices (Coury, 2004), several studies (Finer et al., 2004; Lin et al., 2005; Park et al., 2009; Seiss et al., 2009) have showed that polymers may be subject to numerous biodegradation processes in the oral cavity (Bettencourt et al., 2010). Biodegradation is the gradual breakdown of materials mediated by living organisms which leads to changes in physical properties. In general, degradation of a polymer is defined as a chain scission process during which polymer chains are cleaved into oligomers and in special cases into monomers (Geurtsen, 1998). In the mouth, degradation is a complex process in which is included disintegration and dissolution of materials in saliva (Santerre et al., 2001). This process was already demonstrated in composite resins by several authors (Munksgaard et al., 1990; Larsen et al., 1991; Hagio et al., 2006).

It is well known that the enzymatic activity of hydrolases in human saliva plays a role on the degradation of composite resin monomers (Geurtsen, 1998; Finer et al., 2004; Lin et al., 2005; Ferracane, 2006). Methacrylate-based polymer networks have numerous ester groups that are subject to chemical and enzymatic hydrolysis in the oral cavity. Once each methacrylate functional group contributes with an ester bond to the polymerized network, methacrylate-based acrylic resins are particularly susceptible to

hydrolysis by salivary esterases (Geurtsen, 1998; Park et al., 2009), including cholesterol esterase, pseudocholinesterase, porcine liver esterase, and acethylcholinesterase (AChE) (Yourtee et al., 2001). Salivary esterase and other oral enzymes have been shown to be able to degrade the dimethacrylate resin matrix, by assuming pendant methacrylate groups (Figure 1), resulting in the production and the liberation of methacrylic acid (MA). Esterase-catalyzed degradation of methacrylate-based dental materials has been documented in solution, in saliva samples and *in vivo* (Park et al., 2009). In contrast to composites, there has been very limited investigation of the biodegradation of acrylic resins in the presence of esterases.

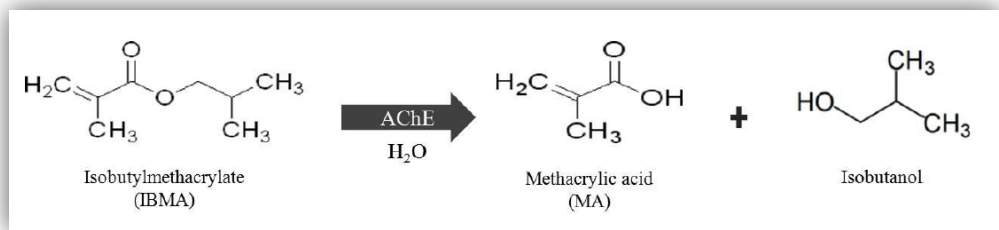

Figure 1 – Representation of the AChE-catalyzed hydrolysis reaction of the IBMA.

While current *in vitro* tests alone are not capable of reproducing these entire complex processes, they can provide a hint of what might be anticipated *in vivo* (Santerre et al., 2001). In order to simulate the oral cavity, the use of enzymatical solutions for degradation analyses has been proposed (Kournetas, 2005).

The main objective of this work is to study the effect of salivary enzyme AChE on the biodegradation of acrylic reline resins. The initial objective is to do an *in vitro* evaluation of the biologic effect of the eluates from acrylic reline resins submitted to AChE, using two colorimetric functional assay, mitochondrial dehydrogenase activity (MTT) and Lactacte dehydrohenase activity (LDH).

Furthermore, this study will try to assess the cytotoxicity of the monomers by using pure compounds of IBMA, MMA, HDMA and their common by-product MA compound, reaching the half maximal inhibitory concentration (IC50) and relating it with the IC50 of the acrylic reline resin liquids. This analysis will try 1) to enlighten the role of the monomers on the cytotoxicity of the eluates from the acrylic reline resins, and 2) to clarify if the cytotoxicity of the reline liquids is related to the respective pure-compounds, once they are mainly composed by them.

2. Materials and Methods

2.1. Chemicals

All the following chemicals were purchased from Sigma-Aldrich Co. (St.Louis, MO, USA): acethylcholinesterase (AChE), phosphate buffered saline (PBS; 0.01 M phosphate buffer, 0.138 M NaCl, 0.0027 M KCl, pH 7.4), Dulbecco´s Modified Eagle´s medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin solution, trypsin, IBMA, MA, HDMA, dimethylsulfoxide (DMSO), Lactate Dehydrogenase Activity assay kit (LDH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) and trypan blue.

MMA was obtained from Merck KgaA (Schuchardt, Germany).

2.2. Cell Culture

Cell culture procedure was adapted from a method previously described (Neves, 2012). Human Adult Dermal Fibroblast Cells (Zen-Bio,Inc, Chapel Hill, PO, USA) were routinely cultured in DMEM with 3.15 g/L of D-glucose, 11.4% FBS, and 1% penicillin-streptomycin solution. The cells were grown on 25 or 75 cm^2 cell culture flasks at 37°C, under an atmosphere containing 5% of CO^2, provided by a balanced-air incubator (Memmert). Cells were incubated at a density of $1x10^4$ cells/cm^2.

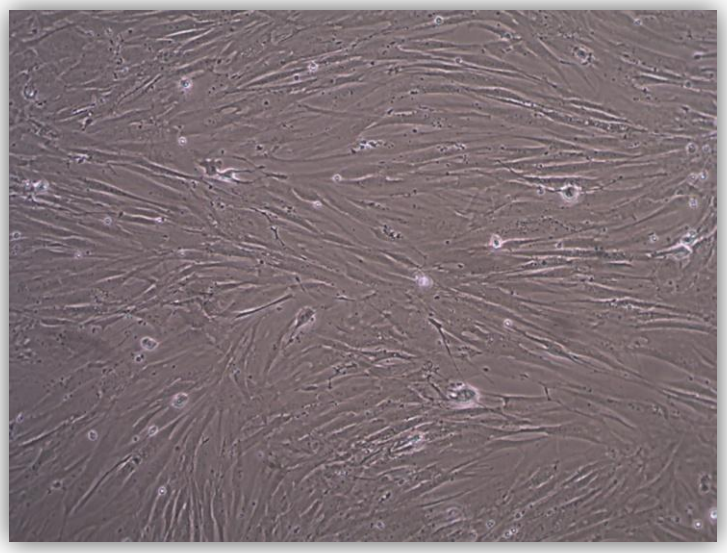

Figure 2 – Representative image of 90-100% cell confluence. Phase contrast microscopy (x20) of human adult dermal fibroblasts.

After cells achieved 90-100% confluence (Figure 2), they were detached from the culture flask and then replated into new culture flasks. In summary, the culture medium was aspirated and the cells were washed with PBS solution, in order to remove all traces of serum. Then, the solution was removed and cells were trypsinized – in order to hydrolyse the inter-cellular adhesion enzymes - by adding 1 mL / 25 cm^2 of 1:9 trypsin/PBS mixture. Fibroblasts were left to trypsinize for 5 minutes at 37ºC. So as to neutralize the trypsin action, stop solution was added using 2 mL/25cm^2 of culture medium. The flask was checked under a microscope (Motic AE 2000) to ensure all cells were free of the flask bottom.

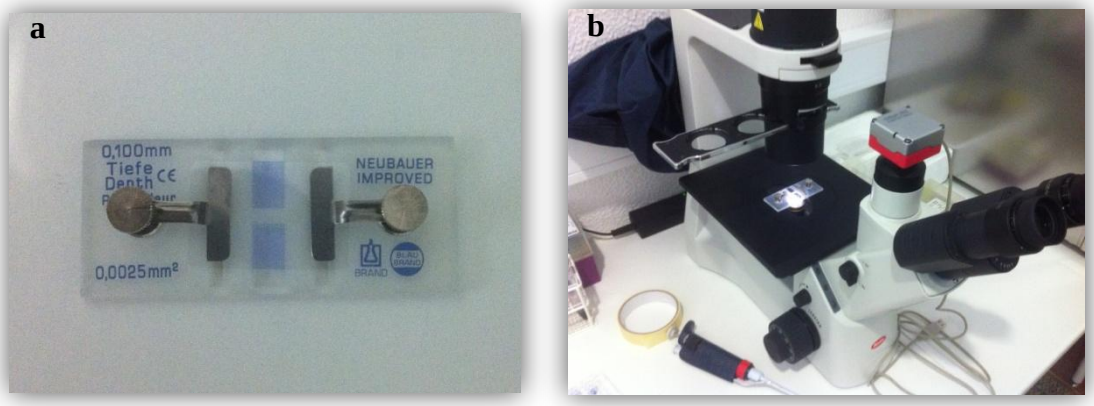

Figure 3 – a) Neubauer camera used for counting the cells on the microscope; b) Motic AE 2000 Binocular Inverted Microscope.

The cellular suspension was centrifuged at 250 x g for 5 minutes at room temperature. The supernatant was aspirated and the cell pellet re-suspended in a volume of DMEM appropriate for cell counting. The cells were counted on a microscope (Motic AE 2000) using a Neubauer camera (Brand), adding 1:1 tryptan blue solution 0.4% that stains dead cells (Figure 3). When viability was higher than 95%, a number of approximately 1×10^4cells per cm^2 were placed in a new culture flask, in a format adequate to the number of cells.

All media, supplements and tissue culture used in this protocol were sterile. Managing passages, trypsinization and preparation of medium or other chemicals was performed in a laminar flow cabinet (Figure 4).

Figure 4 – Laminar flow cabinet to work under sterile conditions.

2.3. Preparation of the test specimens

The three autopolymerized materials included two examples of direct reline resins, Kooliner (GC America Inc, Alsip, Illinois, USA) and Ufi Gel Hard (Voco GmbH, Cuxhaven, Germany), composed of pre-polymerized PEMA powder particles and the monomers IBMA or HDMA, respectively, in the liquid form (Arima et al., 1995, 1996) and one indirect reline resin - Probase Cold (Ivoclar Vivadent AG, Liechtenstein), which represents a PMMA based material which has MMA as the monomer (Table 1).

Product	Manufacturer	Batchnumber	P/L ratio	Composition	Curing Cycle
Kooliner (K)	GC AmericaInc., Alsip, Illinois, USA	1007201 (P) 1008101 (L)	1.4/1	P: PEMA L: IBMA	10 min
Ufi Gel Hard (U)	VocoGmbH, Cuxhaven, Germany	1133100 (P) 1134070 (L)	1.77/1	P: PEMA L: HDMA	7 min
Probase Cold (P)	Ivoclar Vivadent AG, Liechtenstein	L49853(P) L43809(L)	1.5/1	P: PMMA L: MMA	15 min 40º, 2-4 bar

Table 1 – Materials under evaluation in the study

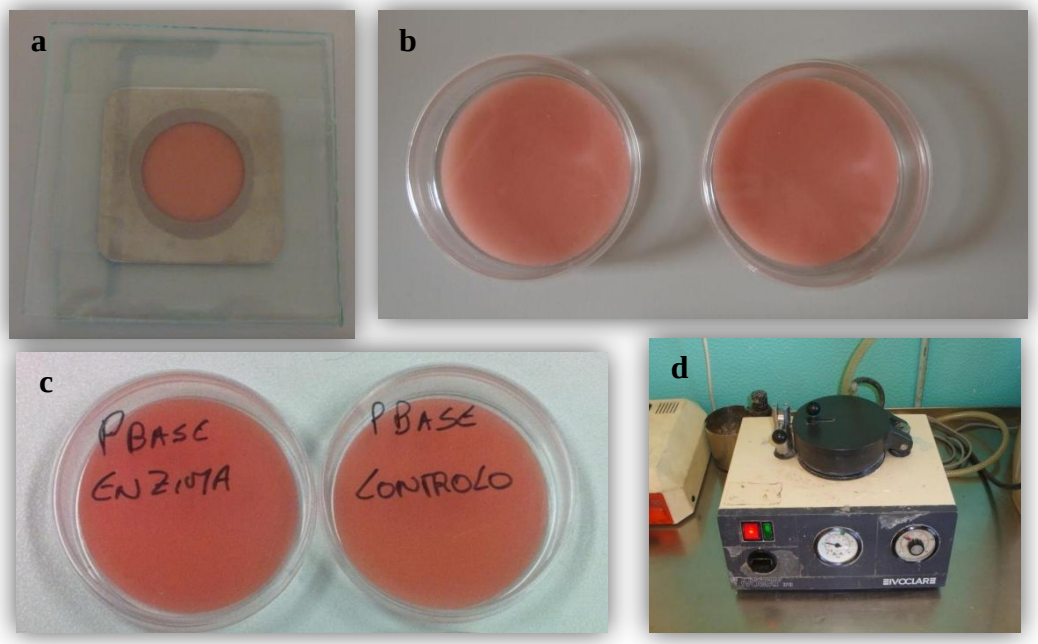

Figure 5 – a) Mixture and mould between polyester sheets and glass plates; b) Examples of two Probase Cold disk shaped specimens; c) Randomly division of two Probase Cold specimens; d) Pressure device Ivomat (IvoclarVivadent, Lichenstein).

Disk-shaped specimens (Figure 5) of each material (n=6) were prepared from three separate mixtures in stainless steel moulds, with an average diameter of 50±0.1 mm and an average thickness of 2±0.01 mm (ISO 20795-1:2008). The total surface area of the specimens (19.64 cm^2) represents a maxillary complete denture bearing area (Minagi et al., 1987) and follows the sample configuration recommended for biological evaluation of biomaterials (ISO 10993-12:2007).

The mould was placed in the centre of a glass plate covered by a polyester sheet. All materials were prepared according to the manufacturers' recommendations and the mixture was placed into the metal mould. A new polyester sheet and glass plate were positioned on top of the mould and the set was maintained under compression, as recommended by ISO for evaluation of biocompatibility of medical devices used in dentistry (ISO 7405:2008).

Direct reline resins were set at 37±2ºC during the recommended polymerization time, in order to simulate the intra-oral polymerization of the material. Polymerization of indirect reline resins was carried out in an Ivomat pressure device (IvoclarVivadent, Lichenstein) for the recommended time, temperature and pressure (Figure 5 d).

2.4. Preparation of the test eluates

The specimens went through 15 minutes of sterilization by UV radiation in dry conditions and at room temperature (Figure 6), as specimens were made in a non-sterile environment (Sheridan et al., 1997, Jorge et al., 2004, Campanha et al., 2006, Jorge et al., 2007). Specimens of each material (n=6) were individually placed in a 55 mm diameter sterilized Petri dish (Nun, InterMed) and randomly divided into two groups: experimental (AChE), immersed in 5 mL of serum-free DMEM with 5 U/mL of AChE and control, immersed only in 5 mL of serum-free culture DMEM. The volume of the medium was selected in order to cover all the surface of each specimen (ISO 10993-12:2007).

Specimens were incubated for 72 h at 37ºC under constant agitation in a mini incubator (Labnet) to allow the soluble components to leach into the medium (ISO 10993-1:2009). Every 24 h, 5 U/mL of AChE was added to AChE specimens, in order to maintain the enzyme activity and serum-free DMEM was added to control specimens, both under sterile conditions in a laminar flow cabinet. The medium without specimens was also incubated as above to serve as the negative control. The protocol of the experimental group was repeated in an enzyme control procedure, with the medium supplemented with 5U/ml AChE without specimens, to test the individually effect of the enzyme in cell viability.

Figure 6 – UV specimens sterilization.

After 72 h, the medium of specimens was collected and 11.4% of fetal bovine serum was added. All specimens' eluate were then diluted in fresh supplemented DMEM as follows: no dilution (100%), 3:4 dilution (75%) and 1:2 dilution (50%), to

check the dose-dependent response of the cultured cells (Figure 3.6). Accuracy is measured by taking into account that the 50 % extract of the test sample should have higher or at least the same cell viability than the 100 % extract; otherwise the test should be repeated (ISO 10993-5:2009).

2.5. Cytotoxicity assay

Cytotoxicity is a primary factor of biocompatibility and is generally determined by *in vitro* cell culture (Att et al., 2009).

The *in vitro* cytotoxicity of these three autopolymerized acrylic reline resins was quantitated by the endpoint of cell viability, MTT reduction assay, and by the release of a soluble cytosolic enzyme Lactate Dehydrogenase (LDH) into the cell culture medium as the marker for membrane damage (Arechabala et al., 1999; Issa et al., 2004).

Cells were inoculated into 96-well tissue culture plates (Sarstedt) at a density of, approximately, $3.2x10^3$ cells/well and incubated at 37°C under a 5% CO^2 atmosphere to allow the cells to attach to the culture dish. After 24 h (correspondent to this cell type doubling period), the necessary subconfluent monolayer was verified using a microscope (Figure 7) and the supernatant was then removed. Cells were then treated for a further 24 h period with 200 µL per well of serial dilutions of the eluates and the test compound solutions (n=8) per combination. Enzyme, negative and positive controls were included in each assay. As positive control, cells were cultured in the medium containing 20% DMSO (ISO 7405:2008). Enzyme and negative controls were explained previously in the preparation of the eluates. After the 24 h-incubation, each plate was examined under a microscope to identify systematic cell seeding errors and undesirable growth characteristics of control and treated cells that can indicate experimental error, leading to the rejection of the assay.

After this examination, the medium was carefully removed from each plate and pipetted to a new vial, to be used later in the LDH assay.

2.5.1. MTT assay

MTT reduction assay is a rapid colorimetric assay, based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) which was first described in 1983 by Mosmann. This test evaluates the mitochondrial function of the

cell since the salt is metabolized by the mitochondrial dehydrogenases of active cells into blue formazan crystals (Jorge et al., 2006; Chaves et al., 2012).

The remaining cells were washed with sterile PBS (37ºC, pH 7.4) to remove non-adherent cells and chemicals that can reduce MTT action and cause false negative results. Then, 200 µL of MTT solution (0.5 mg/mL of MTT in culture medium) was added to each well. The cells were incubated for a further period of 2.5 h at 37ºC and then the MTT solution was discarded and cells were carefully washed with PBS.

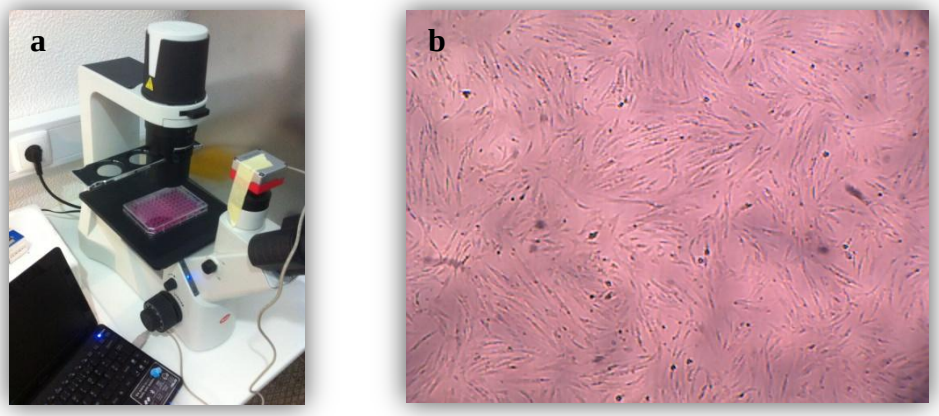

Figure 7 – a) Motic AE 2000 Binocular Inverted Microscope; b) Representative image of subconfluent monolayer (x10).

A soluble solvent, DMSO (200 µL), was added to each well to dissolve the formazan crystals (Figure 8), and absorbance was read at a wavelength of 595 nm with a spectrophotometer (Anthos Zenyth 3100). The background absorbance was measured using a reference wavelength of 690 nm.

2.5.2. LDH assay

Lactate dehydrogenase is an oxidoreductase enzyme that catalyses the interconversion of pyruvate and lactate. The increase of the LDH activity in culture supernatant is proportional to the number of lysed cells (Arechabala et al., 1999). LDH catalyses the reduction of NAD+ to NADH in the presence of L-lactate. The LDH reduction is specifically detected by colorimetric (490nm) assay.

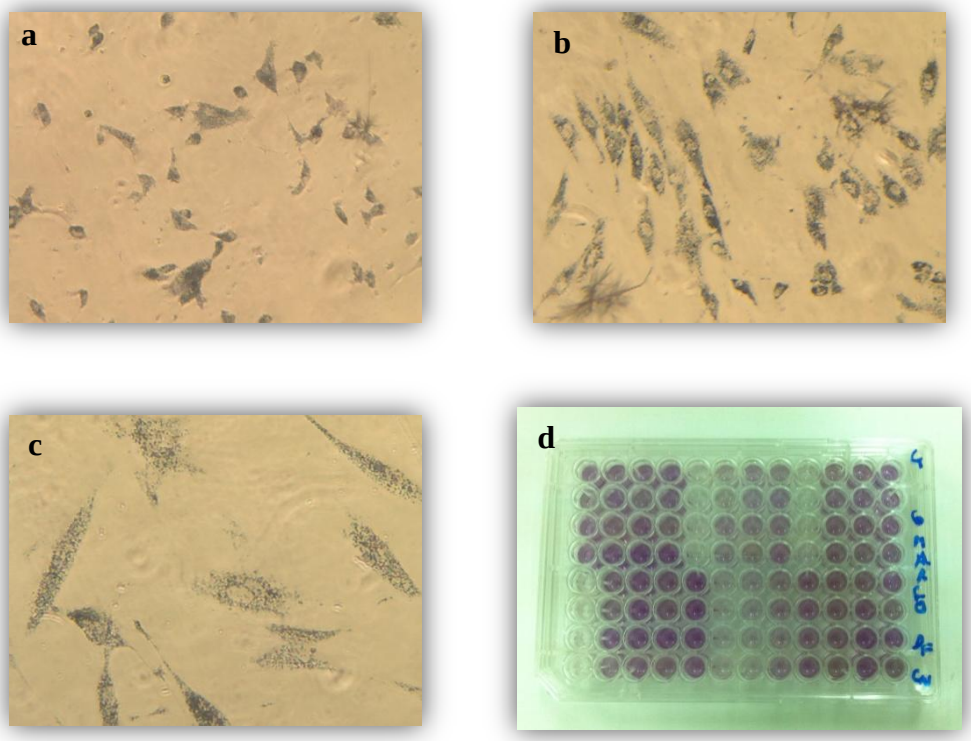

Figure 8 - a-c) Representative image of formazan crystals (x20, x40 and x100, respectively); d) Representative 96 well-plate before absorbance reading.

The medium removed earlier, from the 96 well plates where cells were submitted to the MTT assay, was then centrifuged at 10.000 x g for 10 minutes. The supernatant (25µL) were moved to a new 96 well plate along with a mixture of 25µL of PBS and 50µl of reconstituted substrate mix already prepared from the LDH Kit. Then, plates were kept for 24 min in a dark room at room temperature. Absorbance was recorded both at 490 and 690nm on a spectrophotometer (Anthos Zenyth 3100). The percentage release of LDH from the treated cells was calculated by comparing it to the release of LDH achieved on the negative control cells.

A decrease in number of living cells results in a decrease in the metabolic activity in the sample (MTT assay) or in an increase of lactate dehydrogenase in the medium (LDH assay). Three independent experiments were performed and eight replicate cultures were used for each test solution and controls in each independent experiment. The mean and standard error of the mean absorbance for each test solution were calculated from the triplicate samples. Results of the colorimetric assays were expressed as percentage of viable cells yielded by the test solutions compared to negative controls. The reduction of viability compared to negative controls is calculated as follows:

$$Viability\ \% = \frac{\text{ODassay} - 690\text{e}}{\text{ODassay} - 690\text{c}} \times 100$$

Where ODassay-690e is the mean value of the measured optical density of the cells incubated to the experimental solution; ODassay-690c is the mean value of the measured optical density of the negative controls. The lower the cell viability value, the higher the cytotoxic potential of the test item is. Cytotoxicity was also rated based on cell viability relative to controls in accordance with ISO-standard 10993-5:2009 as non-cytotoxic > 75% cell viability; slightly cytotoxic 50-75% cell viability; moderately cytotoxic 25-50% cell viability; and severely cytotoxic <25% cell viability (ISO 10993-5: 2009).

2.6. Preparation of the test compounds solutions and acrylic reline resin liquids

Standard pure compounds MMA, IBMA, HDMA that are expected to be leached from the three acrylic reline resins and the degradation by-product MA were tested for cytotoxicity, as well the respective acrylic reline resins liquids. At least seven concentrations of each compound were diluted in DMEM supplemented with ethanol, in order to dissolve the high lipophilic samples. The final concentration of ethanol in each sample was ≤0.3%. The prepared concentrations were based on the studies of Chaves et al. (2010) and Lai et al. (2004) in order to obtain the IC50 of each.

These samples were measured only by the MTT assay. IC50 was determined using a non-linear regression of Dose-Response – Inhibition type [log(inhibitor) vs normalized response – Variable slope].

2.7. Statistical Analysis

The Kolmogorov-Smirnov test was used to assess the normality of cell viability variable. Since the test rejected the null hypothesis of normality of the distribution, non-parametric tests were used. Mann-Whitney tests were used to compare cell viability between control and experimental groups. To compare between materials, test compounds and dilutions, Kruskall-Wallis was used, followed by post testing Tukey multiple comparison. P values ≤ 0.05 were considered significant. All analyses were performed with the SPSS statistical package (version 20, SPSS Inc. Chicago IL).

3. Results

3.1. Cytotoxicity of the eluates

3.1.1. MTT Assay

There was no difference ($p<0.001$) in cell viability (Figure 9) when the fibroblast cells were exposed to medium alone (negative control) or medium containing AChE (enzyme control).

Figure 9 shows that: 1) No cytotoxicity was observed for Probase Cold specimens; 2) Kooliner and Ufi Gel Hard specimens showed reduction of viability compared with the 100% negative control group ($p<0.001$); 3) ~90% decrease in cell viabilibity for Kooliner specimens, 4) ~51% decrease in cell viabilibity for Ufigel Hard specimens. Differences between the three materials were statistically significant ($p<0.001$).

Kooliner specimens, both control and experimental groups, proved to be severely cytotoxic for the fibroblast cells. Kooliner specimens submitted to treatment with AChE showed a slight increase of cell viability (18.8±9.2%) compared with the control specimens (9.0%±4.9%, $p<0.001$). For Ufi Gel Hard specimens, the cell viability of the experimental group submitted to AChE (72.5%±12%) showed also an increase compared with the specimens incubated only in the culture medium (48.3%±15.8%, $p<0.001$) as recorded on Figure 9.

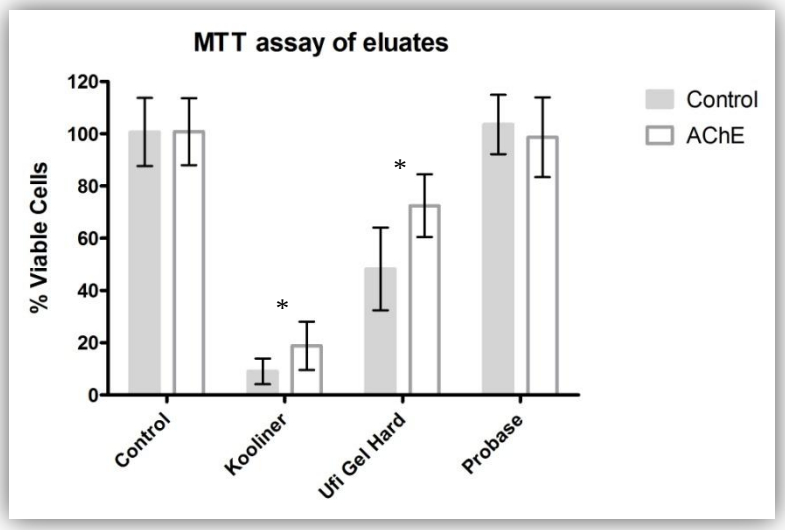

Figure 9 - Effect of acetylcholinesterase on the cytotoxicity of 3 reline resins expressed as percentage of viable fibroblast present after exposure compared with the negative control group set as 100%; * means significant differences between experimental and control groups.

Data also indicated a dose-dependent effect on cytotoxicity for the different dilutions of Kooliner and Ufi Gel Hard eluates, as shown on Figure 10.

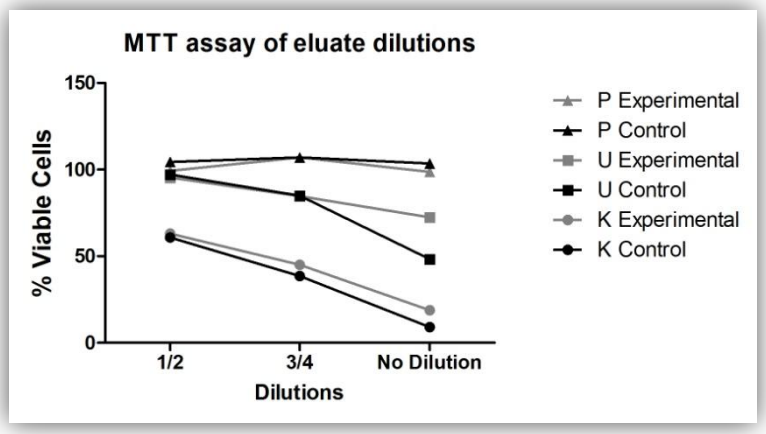

Figure 10 – Cytotoxicity of Kooliner (K), Ufigel Hard (U) and Probase Cold (P) dilutions expressed as percentage of viable fibroblasts present after exposure compared with the negative control group set as 100%.

3.1.2. LDH assay

As observed in the MTT assay, fibroblast cells incubated with culture medium with AChE and without exposure to material (enzyme control), didn't show differences when compared with the medium alone (negative control) ($p<0.001$). However, neither control nor experimental groups demonstrated differences when compared with control groups (Figure 11).

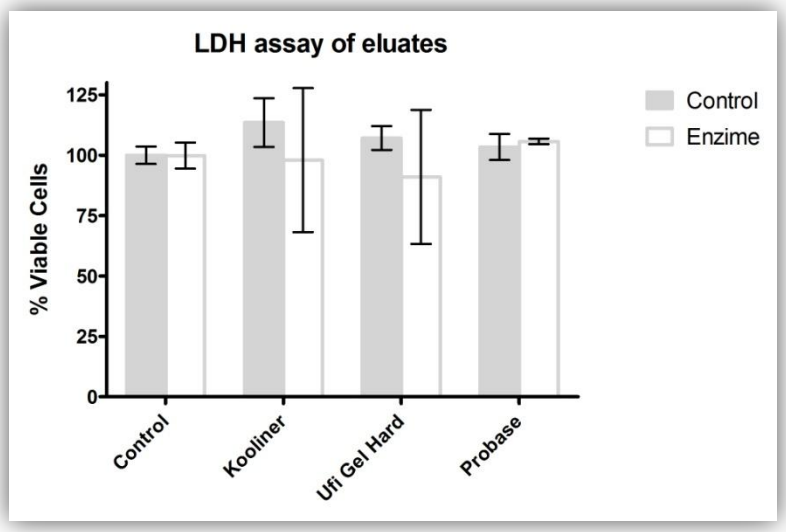

Figure 11 - Effect of acetylcholinesterase on the cytotoxicity of 3 reline resins expressed as percentage of viable fibroblast present after exposure compared with the negative control group set as 100%.

3.2. Cytotoxicity of the test compounds solutions

Treatment with various resin liquids and methacrylate-based monomers impaired the viability of primary dermal fibroblasts cells in a dose dependent manner (Figure 12-17). Non-linear regression of Dose-Response – Inhibition type [log(inhibitor) vs normalized response – Variable slope] was used to predict the IC50 of monomers and resin liquids. Figures 12-14 exhibits a good fit of calculated curve to observed points. It is observed an S shaped curve.

Approximately 50% of the cellular viability was affected when 0,2715 mmol/L of HDMA, 3,521 mmol/L of IBMA, 31,88 mmol/L of MA was used (Appendix 8.4. IC50 Determination Table). MMA showed no cytotoxicity at the concentrations used, and so it was not possible to reach IC50.

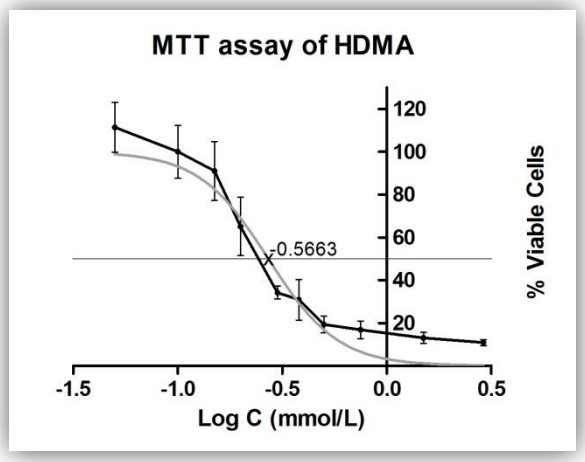

Figure 12 - Cellular viability as determined by MTT assay. Percentage of cellular viability of cells treated with increasing concentrations of HDMA for 24h. Results are expressed as the mean ± s.d. IC50 determination.

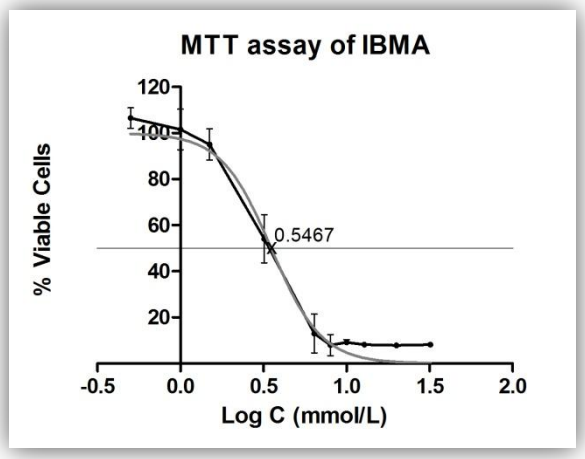

Figure 13 - Cellular viability as determined by MTT assay. Percentage of cellular viability of cells treated with increasing concentrations of IBMA for 24h. Results are expressed as the mean ± s.d. IC50 determination.

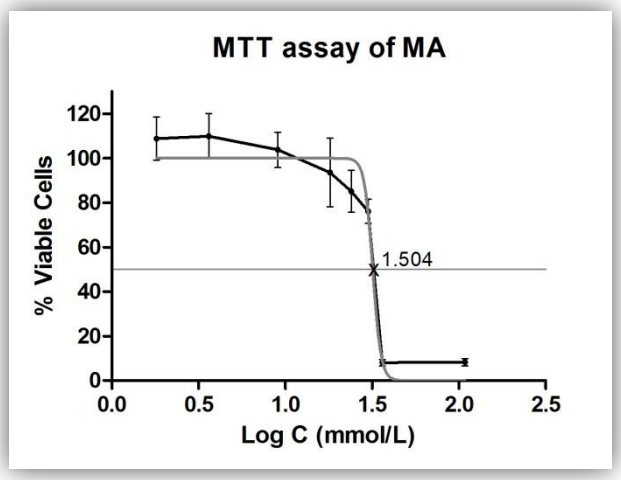

Figure 14 - Cellular viability as determined by MTT assay. Percentage of cellular viability of cells treated with increasing concentrations of MA for 24h. Results are expressed as the mean ± s.d. IC50 determination.

Considering the IC50 of the residual monomers, HDMA showed to be the most cytotoxic compound among the chemicals tested. The cytotoxicity decreased in order of HDMA>IBMA>MA for the human dermal fibroblasts.

3.3. Cytotoxicity of the acrylic reline resins liquids

Figures 15-17 exhibit point-to-point curves of resins liquids and respective monomers. The IC50 of the reline resin liquids is obtained with, respectively, 0,2587 mmol/L of Ufi Gel Hard, 6,496 mmol/L of Kooliner and 7,124 mmol/L of Probase Cold. Ufi Gel Hard liquid appeared to be the most cytotoxic among the various resin liquids examined (Figure 15-17).

The curve shape of the monomers, on the figure 15 and 16, matches with the one of the resins liquids, exhibiting similar behavioral. Figure 17 shows that the monomer doesn’t exhibit a cytotoxic behavioral at the concentrations used, while comparable concentrations of probase liquid reveals a regular S shaped curve.

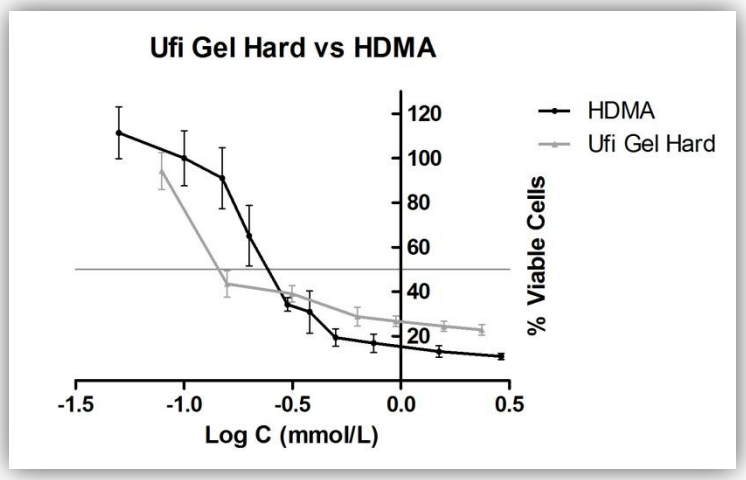

Figure 15 – Analysis of curves and comparison of Ufi Gel Hard liquid and HDMA IC50.

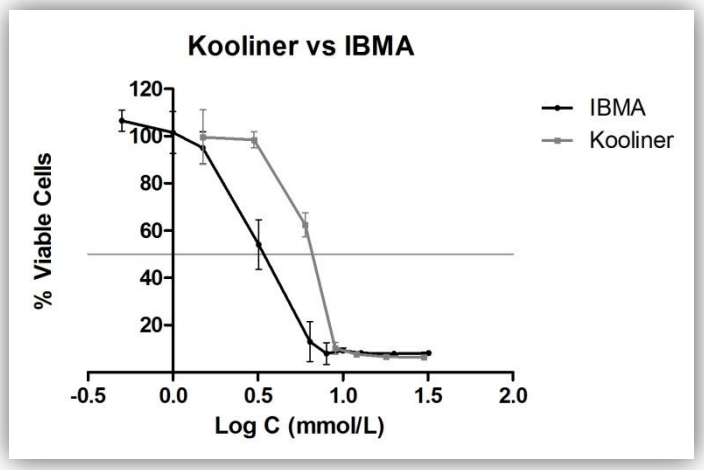

Figure 16 - Analysis of curves and comparison of Kooliner liquid and IBMA IC50.

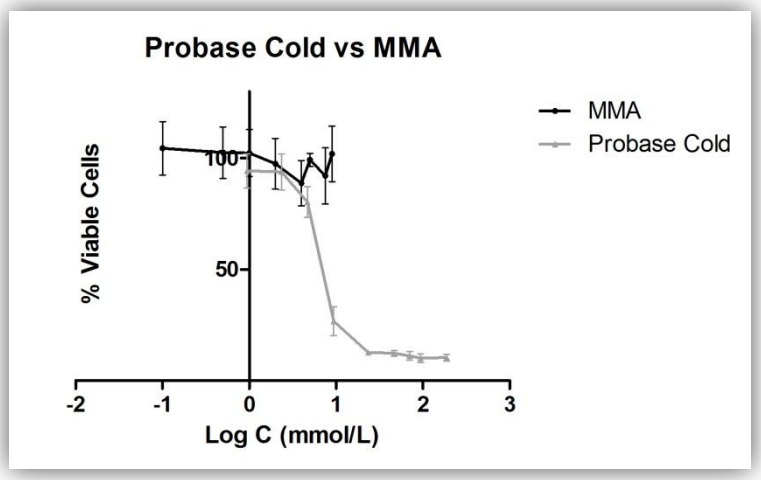

Figure 17 - Analysis of curves and comparison of Probase Cold liquid and MMA IC50.

4. Discussion

The use of hard chairside autopolymerizing resins for the direct relining of dentures has gained popularity as they are easy to manipulate and need no laboratory procedures (Urban et al., 2009; Chaves et al., 2010). However, cases of local chemical irritation and allergic reactions among acrylic-based prosthesis wearers have been documented (Arima et al., 1996; Huang et al., 2001; Chaves et al., 2012). These reactions are believed to be caused by the release of monomers from the polymer network (Ebadian et al., 2008; Ebrahimi Saravi et al., 2012). Therefore, it is important to know the level of toxicity of biomaterials used for relining as well as the structure–toxicity relationship of acrylic resin components. *In vitro* cytotoxicity tests are a necessary screening step in the evaluation of new materials used in humans. In addition, those methods are simple, reproducible, cost effective, and suitable for the evaluation of basic biologic properties of dental materials (Huang et al., 2001).

In the present study, cytotoxicity of acrylic reline resins, submitted to a salivary esterase, was assessed by using two separate and unrelated colorimetric functional assays, MTT and LDH, in human primary fibroblasts.

Cell selection was based on the fact that human fibroblasts are found to be more sensitive than epithelial cells, for evaluation with eluates. Furthermore, they are exposed to denture base resins when ulceration of epithelium occurs after denture placement (Huang et al., 2001 and 2002). Also, the toxic components may be capable of affecting tissue sites distant from the resin contact area as they are diffusible in an aqueous environment. This may be a particular problem for patients having mucosa that is infected, inflamed, lacerated or fragile as a result of nutritional problems or concurrent medications. Thus, large areas of the oral mucosa may be exposed to these toxic components over an extended period of time (Lefebvre et al., 1994). Moreover, fibroblasts have high growth activity (Huang et al., 2001 and 2002, Chaves et al., 2012) a well characterized cell system and biological responses, and they are easily maintained in typical laboratory conditions.

The MTT colorimetric method has been widely used to estimate the cytotoxicity of dental polymers. In addition, it was observed that the spectrophotometric evaluation of the solubilized formazan dye is fast, objective and has the least variation among several others (Chaves et al., 2010).

The LDH assay usually operates as a confirmation test and as a biomarker for membrane damage, and consequently, cell death. It is based on the measurement of activity of lactate dehydrogenase which is a stable enzyme normally found in the cytosol but rapidly released into the supernatant upon damage of plasma membrane.

In this study, the exposure of fibroblasts to direct reline resins, Kooliner and Ufi Gel Hard eluates resulted in a significant suppression of fibroblastic function, characterized by supressed mitochondrial activity. This result is in accordance with the study of Neves-2012. On the contrary, a previous study on cytotoxicity of acrylic resins (Campanha et al., 2006) found that, through MTT assays, direct reline resins eluates did not show any toxic effects on the L929 mouse lung fibroblasts cell line. These results could be explained by the distinct type of cells used in the studies. Huang et al. (2001) demonstrated that specific cell types react differently to the same dental materials. Most tests are done in transformed L929 mouse cells (Cimpan et al., 2000, Jorge et al., 2004, Campanha et al., 2006, Jorge et al., 2007) as the model of cell response, but normal diploid cells, present in the primary cultures, can respond differently to cytotoxic challenge. Several authors showed that primary cells have greater sensitivity than transformed lines when testing various biomaterials used in dentistry (Feigal et al., 1985; Huang et al., 2001). Primary cultures have a more normal phenotype and they correlate to an *in vivo* response more accurately, so they can be considered to be more appropriate for testing toxicity of materials for human use (Neves, 2012).

Even though several previous studies had showed that indirect autopolymerized eluates were cytotoxic to fibroblasts (Tsuchiya et al., 1994; Lefebvre et al., 1995; Schuster et al., 1995; Sheridan et al., 1997; Cimpan et al., 2000), the present study did not find cytotoxicity on eluates of Probase Cold. This can be explained by the recommended pressure and temperature treatment during the polymerization that the indirect reline resin used in our study - Probase Cold - suffered, in opposition to the polymerization at room temperature, as advised by the manufacturers of autopolymerizing resins used in previous studies (Cimpan et al., 2000; Huang et al., 2001). Immersion in hot water can promote further polymerization and release of residual components potentially toxic to cells, prior to the incubation of the specimens with culture medium.

Previous findings also suggest that more severe tissue reactions may occur at higher concentrations of monomers, showing that cytotoxicity of resins is dose-

dependent. In the present study, Kooliner showed a higher cytotoxic effect than Ufi Gel Hard in control specimens. The fact that Kooliner showed a higher percentage of residual monomer content than Ufi Gel Hard (Urban et al., 2007 and 2009; Neves, 2012) could explain this difference. Kedjarune et al. (1999) found that the more monomer added to the mixture the greater the amount of RM and therefore the higher the potential for cytotoxicity. The powder/liquid ratio is lower in the Kooliner specimens, which means that for the same amount of powder it takes more liquid in the mixture than Ufi Gel Hard, and consequently, more monomer.

In this study, Ufi Gel Hard eluates suppressed around 51% cell viability. In spite of the severe cytotoxicity potential of HDMA, defended by Chaves et al. (2010) and Neves in 2012, the low levels of RM content of this resin promoted only moderately cytotoxic effects over the fibroblast cells.

In contrast, the highly toxic effect of Kooliner eluates (~10%) cannot be explained solely by a higher percentage of residual IBMA content of specimens. This may also be due to differences in quantity and quality of other potentially toxic compounds (Koda et al., 1989 and 1990; Cimpan et al., 2000) that may be leached from the resins as cross linking agents, plasticizers like ethylenoglycoldimethacrylate (EGDMA) or tetramethylene dimethacrylate (TMDMA) (Vallittu et al., 1998), pigments, degradation by-products like MA and newly formed formaldehyde (Ruyter 1980).

Furthermore, peroxidation of cellular lipids by benzoyl peroxide (BPO) commonly used as a polymerization initiator in denture resins, may contribute to the toxicity of the materials (Masuki et al., 2007). It has been demonstrated that free radicals resulting from the decomposition of BPO during polymerization are released and that this is a long-lasting event. Free radicals are highly reactive against all biological molecules and are able to injure cells and tissues namely by peroxidation of cellular lipids (Cimpan et al., 2000; Masuki et al., 2007). These findings might explain the cytotoxic potential of the Kooliner eluates tested. In addition, potential synergetic effects of the leachable chemicals should also be considered (Neves, 2012).

The biological and toxicological effects of biomaterials depends on their behaviour in the oral environment, thus the mimetization of the medium is crucial. The quantity and type of leachable compounds of acrylic resins depends on the medium composition (Ferracane et al., 1990).

Acetylcholinesterase is present in the oral cavity and can catalyse the hydrolysis of ester compounds such as the leachable monomers of reline resins. Degradation products of salivary enzymes were already extensively studied, mostly in composite resins. In this study, a salivary enzyme was added to the medium in order to study the degradation process caused by an enzyme and its effects on cytotoxicity.

Cells incubated with eluates treated with acethylcholinesterase changed their cytotoxic response to eluates from direct reline resins. The enzyme did not change the non-cytotoxic effect of the indirect reline resin Probase Cold. AChE experimental specimens of Kooliner and Ufi Gel Hard showed an increase of cell viability, compared to control specimens.

The increase of cell viability of experimental Kooliner specimens (submitted to the enzyme) can be explained by the hydrolysis of IBMA promoted by the enzymatic reaction. As proposed by Neves (2012), MA was found to be a product of this reaction, but the lower cytotoxic potential of MA comparing to IBMA demonstrated by this study and before by several groups (Chaves et al., 2010; Neves, 2012) can explain the reduction of the cytotoxicity. In addition, MA proved to be a very unstable compound in aqueous solutions (Baker et al., 1988).

Quite the opposite, the slender increase of cell viability of AChE experimental Ufi Gel Hard specimens could not be related to the enzymatic reaction since HDMA was found to be resistant to AChE (Neves, 2012). However, levels of MA, obtained in other studies, reveal that AChE promoted production of MA by hydrolysis of other monomers than HDMA that can be present in Ufi Gel Hard specimens (Neves, 2012).

In the present study, results of the dilutions of eluates showed an increase of cell viability in a dose dependent manner. This results also demonstrated the accuracy of the MTT assay, since 50% dilutions showed greater values of cell viability than maximum concentration ones (ISO 10993- 5:2009).

Even though the exposure of fibroblasts to direct reline resins, Kooliner and Ufi Gel Hard eluates, resulted in a significant suppression of fibroblastic function, it didn’t appear to cause membrane damage, since the LDH assay didn´t reveal cytotoxicity results.

Although there was weak cell membrane damage by acrylic reline resin eluates, it would be important to understand other mechanisms of cellular unviability like genotoxic/mutagenic activity by such agents.

Besides concentration, the chemistry of the compounds is an important characteristic that determines their cytotoxicity degree. Eluate studies provide important and realistic data regarding the toxicity of different formulae of reline resins although it does not identify the role of each specific substance released.

In previous studies (Neves, 2012), quantitative determination of residual compounds released from different reline resins (Kooliner, Ufigel Hard and Probase Cold) was made by HPLC (High Perfomance Liquid Chromatography). The following compounds were quantified: IBMA (Kooliner), 1,6-HDMA (UfiGel Hard), MMA (Probase Cold) and the degradation by-product MA.

Based on the results of this study, corresponding ranges of concentrations of each of these compounds were used in this work, until double the maximum concentration obtained, to test for cytotoxicity.

Previous studies found that HDMA is an extremely cytotoxic monomer (Neves, 2012), due to its high lipophilicity, promoting a strong interaction with cell membranes with consequent suppression of cell growth and proliferation (Atsumi et al., 2006; Chaves et al., 2010) and induction of apoptosis (Schuster et al., 1995, Yoshii, 1997). IBMA monomer also exhibited highly cytotoxic effect on L929 cells (Campanha et al., 2010).

Pure compounds at concentrations measured in eluates extracted from Kooliner and Ufi Gel Hard specimens (Urban et al., 2009) were proved to be cytotoxic to L929 fibroblasts, using MTT and DNA synthesis assay (Chaves et al., 2010). However, the present study obtained different results, with compounds showing no cytotoxic effects when exposed to human primary fibroblasts, at concentrations found to be leached to the oral environment. This can be explained by the insolubility of the compounds in an aqueous environment that may lead to a reduced diffusion through the culture medium to the cells. Additionally, the high volatility of the compounds may result in a much reduced time of exposure of cells to such monomers. It can be speculated that if monomers reveal high lipophilicity and volatility the probability of causing local chemical reactions is very low.

As the monomers didn't exhibit cytotoxic behavior at the concentrations leached from the reline resins, it is important to understand the role of the compounds on the cytotoxicity of the reline resins liquids, achieving both IC50 in order to predict if there is another component responsible for the cytotoxicity of the resin eluates. For that matter, this study evaluated the cytotoxicity of various relining resin liquids and their major components effect on primary dermal fibroblasts cells. To the best of our knowledge, there isn't any data available yet about the IC50 of such monomers in primary dermal fibroblasts.

Among the tested materials, the Ufi Gel Hard liquid and its monomer, 1,6-HDMA, showed the greatest toxic effects, whereas MMA had the smallest effect.

Both Kooliner liquid and IBMA showed moderate cytotoxicity. The intent of incorporating IBMA into dental polymers was to reduce water absorption by denture bases (Lai et al. 2004). However, the cytotoxicity of butyl methacrylate is believed to be due to its lipophilicity. This finding was supported by an investigation on the cytotoxic effects of six methacrylates with alkyl substituents on cell viability. The IBMA, which has a longer alkyl chain than MMA, may have higher lipophilicity and also demonstrated higher cytotoxicity (Yoshii, 1997).

The presence of IBMA and 1,6-HDMA explains the cytotoxic effects observed for Kooliner liquid and Ufi Gel Hard liquid, respectively. However, even in higher concentrations, MMA showed no cytotoxicity effect on fibroblasts (Figure 17). MMA alone cannot completely explain the effects of Probase Cold liquid on the viability of cells. The effects of Probase Cold liquid in the cellular viability can be explained by the hydrolysis of the monomer MMA in MA, being the latest more cytotoxic, according to our results. Besides that, Probase Cold liquid is composed by a plasticizer tetramethylene dimethacrylate which can be responsible for such cytotoxicity. Even though in lesser percentage, the effect of this compound on the fibroblasts viability is unknown.

It is important to emphasize that the results of cytotoxicity tests present limitations with regard to their applicability to clinical situations. Findings for either *in vitro* tests or those performed *in vivo* cannot be extrapolated to the clinical setting. Nevertheless, such tests are important because vital information with respect to the biological behavior of dental materials and their components can be obtained. Further

studies are necessary to identify all the individual toxic components of the acrylic reline resins that leach into saliva but above all the products of the degradation process.

It was attempted to simulate oral conditions in terms of activity of esterase. However, under oral conditions, there is a constant flow of saliva of 0.5–1 ml per minute, the composition of saliva varies and not all parts of the lining material may be in contact with saliva. These differences should be taken into consideration when interpreting the results (Munskgaard, 2005).

Though the unreliability of the artificial saliva compounds, it was demonstrated that a certain compound was more capable to provoke filler leaching from experimental dental composites than distilled water. This imposes questions whether distilled water or artificial saliva or eventually another solution may lie closer to the clinical situation (Kournetas, 2005). Others authors (Kedjarune et al. 1999; Jaffer et al. 2002; Lin et al. 2005; Hagio et al. 2006) tried to surpass this barrier collecting unstimulated human whole saliva and using the supernatant.

In vitro cytotoxicity tests have been playing a central role in testing for biocompatibility of chemicals, in the literature. However, it would be important also to make a morphological analysis by flow cytometry in order to evaluate the cytopathogenic effects of denture base resins because apoptosis and necrosis have different biological significance (Cimpan et al. 2000; Lai et al. 2004; Masuki et al. 2007). *In vivo*, apoptotic cells are removed by phagocytes and thus, an inflammatory response is prevented. Necrosis, on the other hand, induces inflammation and injuries to the surrounding tissues. Therefore, if the compounds eluted from a denture induce apoptosis, then the tissues that come in contact with the denture would more likely adapt to modifications induced by it, whereas if they induce necrosis, the consequent inflammatory phenomena can induce severe tissue reactions.

Notwithstanding, the actual mechanism of the cytotoxicity of acrylic resins or, for that matter, monomers, is not well known. Other studies have already demonstrated that acrylic resins are capable of inhibiting DNA synthesis (Yang et al., 2003; Ishikawa et al., 2006), in addition to lipid metabolism (Schuster et al., 1995; Lai et al., 2004), cytokine production, and inhibition of cell viability via mitochondrial activity (Huang, 2001). Although not clear yet, there are two known mechanisms underlying the adverse effects of resin materials: genetic damage and an oxidative stress caused from an imbalance between reactive oxygen species (ROS) and anti-oxidant redox defensive

system. Monomers released from resin materials above a certain concentration cause DNA damage that results in a delay or arrest of a cell cycle. Resin monomers increase intracellular ROS, as represented by hydrogen peroxide, superoxide anions, and hydroxyl radicals, and subsequently reduce the intercellular level of antioxidant molecules like glutathione (GSH), a direct ROS scavenger. The increased ROS after the GSH depletion may induce cytotoxicity by modulating the signaling pathways leading to cell death. In addition, ROS may directly damage the cellular structure (Att et al. 2009).

A limitation of this work is that the use of surface area of the specimens is believed to give higher inaccuracy than the use of their mass. Therefore, in future studies the ratio between the mass of the specimens and the volume of the extraction medium should be used, as recommended by the ISO standard.

5. Conclusions

Within the limitations of this study, the main conclusions of this thesis are:

- ✓ The indirect reline resin Probase Cold eluate demonstrated no cytotoxicity effect to human fibroblasts.
- ✓ Both direct reline resins revealed cytotoxicity to human fibroblasts: Kooliner specimens showed to be severely cytotoxic and Ufi Gel Hard specimens moderately cytotoxic.
- ✓ Incubation with acetylcholinesterase did not change the non-cytotoxic effect of Probase Cold.
- ✓ Incubation with AChE caused a slight increase on cell viability of both direct reline resins (Kooliner and Ufi Gel Hard).
- ✓ Considering the IC50 of the residual monomers, the cytotoxicity decreased in order of HDMA>IBMA>MA for the human dermal fibroblasts. MMA showed no cytotoxicity at the concentrations used.
- ✓ At the concentrations found to be leached in the oral cavity, based on the results of previous studies, no cytotoxic effects of monomers were observed when exposed to human primary fibroblasts.
- ✓ The cytotoxicity of the Ufi Gel Hard and Kooliner liquid is similar to that found on the HDMA and IBMA alone experiments.
- ✓ MMA cannot explain the effects of Probase Cold liquid on the fibroblasts viability.

6. References

1. Aalto-Korte K, Alanko K, Kuuliala O, Jolanki R. Methacrylate and acrylate allergy in dental personnel. Contact Dermatitis 2007; 57:324-330.

2. Arechabala B, Coiffard C, Rivalland P, Coiffard LJM, de Roeck-Holtzhauer Y. Comparison of cytotoxicity of various surfactants tested on normal human fibroblast cultures using the neutral red test, MTT assay and LDH release. J Appl Toxicol 1999; 3:163–165.

3. Arima T, Murata H, Hamata T. Analysis of composition and structure of hard autopolymerizing reline resins. J Oral Rehabil 1996; 23:346-352.

4. Atsumi T, Fujisawa S, Tonosaki, K. (Meth)acrylate monomer-induced cytotoxicity and intracellular Ca2+ mobilization in human salivary gland carcinoma cells and human gingival fibroblast cells related to monomer hydrophobicity. Biomaterials 2006; 27:5794-5800.

5. Att W, Yamada M, Kojima N, Ogawa T. N-Acetyl cysteine prevents suppression of oral fibroblast function on poly(methylmethacrylate) resin. Acta Biomater 2009; 5:391-398.

6. Autian J. Structure- Toxicity Relationships monomers. Environ Health Perspect, 1975; 11:141-152.

7. Aydin AK, Terzioglu H, Akinay AE, Ulubayram K, Hasirci N. Bond strength and failure analysis of lining materials to denture resin. Dent Mater 1999; 15:211-218.

8. Azevedo A, Machado AL, Vergani CE, Giampaolo ET, Pavarina AC. Hardness of denture base and hard chair-side reline acrylic resins. J Appl Oral Sci. 2005 Sep;13:291-295.

9. Azzarri MJ, Cortizoa MS, Alessandrini JL. Effect of the curing conditions on the properties of an acrylic denture base resin microwave-polymerised. J Dent 2003; 31:463-468.

10. Baker S, Brooks SC, Walker DM. The release of residual monomeric methyl methacrylate from acrylic appliances in the human mouth: an assay for monomer in saliva. J Dent Res 1988; 67:1295-1299.

11. Bayraktar G, Guvener B, Bural C, Uresin Y. Influence of polymerization method, curing process, and length of time of storage in water on the residual methyl methacrylate content in dental acrylic resins. J Biomed Mater Res B Appl Biomater 2006; 76:340-345.

12. Bettencourt AF, Neves CB, Almeida MA, Pinheiro LM, Oliveira SA, Lopes LP, Castro MF. Biodegradation of acrylic based resins: A review. Dent Mater 2010; 26: e171-e180.

13. Bohnenkamp DM. Traumatic stomatitis following an intraoral denture reline: a clinical report. J Prosthet Dent 1996; 76:113-114.

14. Budtz-Jorgensen E. Prosthodontics for the elderly: diagnosis and treatment.1999. Quintessence Publishing Co, Inc.

15. Bural C, Aktas E, Deniz G, Unlucerci Y, Bayraktar G. Effect of leaching residual methyl methacrylate concentrations on in vitro cytotoxicity of heat polymerized denture base acrylic resin processed with different polymerization cycles. J Appl Oral Sci. 2011 Aug;19:306-312.

16. Campanha NH, Pavarina AC, Giampaolo ET, Machado AL, Carlos IZ, Vergani CE. Cytotoxicity of hard chairside reline resins: effect of microwave irradiation and water bath postpolymerization treatments. Int J Prosthodont 2006; 19:195-201.

17. Celebi N, Yuzugullu B, Canay S, Yucel U. Effect of polymerization methods on the residual monomer level of acrylic resin denture base polymers. Polym Adv Technol 2008; 19:201-206.

18. Chaves CA, Machado AL, Carlos IZ, Giampaolo ET, Pavarina AC, Vergani CE. Cytotoxicity of monomers, plasticizer and degradation by-products released from dental hard chairside reline resins. Dent Mater 2010; 26:1017- 1023.

19. Chaves CA, Machado AL, Vergani CE, Souza RF, Giampaolo ET. Cytotoxicity of denture base and hard chaiside reline materials: a systematic review. J Prosthet Dent 2012; 107:114-127.

20. Cimpan MR, Cressey LI, Skaug N, Halstensen A, Lie SA, Gjertsen BT, Matre R. Patterns of cell death induced by eluates from denture base acrylic resins in U-937 human monoblastoid cells. Eur L Oral Sci 2000; 108: 59-69.

21. Cooper SL, Visser SA, Hergenrother RW, Lamba NK. Classes of materials used in Medicine: Polymers. In: Biomaterials Science- An introduction to materials in medicine. Ratner B, Hoffmen A, Schoen FJ, Lemons JE. 2nd edition. Elsevier Academic Press, London UK. 2004

22. Coury A. Degradation of materials in the biological environment: chemical and biochemical degradation of polymers.In: Biomaterials Science- An introduction to materials in medicine. Ratner B, Hoffmen A, Schoen FJ, Lemons JE. 2nd edition. Elsevier Academic Press, London UK. 2004.

23. Cucci ALM, Rached RN, Giampaolo ET, Vergani CE. Tensile bond strengths of hard chairside reline resins as influence by water storage. J Oral Rehabil 1999; 26:631-634.

24. Douglas C, Shih A, Ostry L. Will there be a need for complete dentures in the United States in 2020? J Prosthet Dent 2002; 87:5–8.

25. Ebadian B, Razavi M, Soleimanpour S, Mosharraf R. Evaluation of tissue reaction to some denture-base materials: an animal study. J Contemp Dent Pract 2008; 1:67-74.

26. Ebrahimi Saravi M, Vojdani M, Bahrani F. Evaluation of cellular toxicity of three denture base acrylic resins. J Dent. 2012; 9:180-188.

27. Ferracane JL, Condon JR. Rate of elution of leachable components from composite. Dent Mater 1990; 6:282-287.

28. Ferracane JL. Hygroscopic and hydrolytic effect in dental polymer networks. Dent Mater 2006; 22:211-222.

29. Finer Y, Jaffer F, Santerre JP. Mutual influence of cholesterol esterase and pseudocholinesterase on the biodegradation of dental composites. Biomaterials 2004; 25:1787-1793.

30. Feigal RJ, Yesilsoy C, Messer HH, Nelson J. Differential sensitivity of normal human pulp and transformed mouse fibroblasts to cytotoxic challenge. Arch Oral Biol 1985; 30: 609-613.

31. Geursten W. Substances released from dental composites and glass ionomer cements. Eur J Oral Sci 1998; 106:687-695.

32. Golbidi F, Asghari G. The level of residual monomer in acrylic denture base materials. Res J Biol Sci 2009; 4:244-249.

33. Gonçalves TS, Morganti MA, Campos LC, Rizzatto SM, Menezes LM. Allergy to autopolymerized acrylic resin in an orthodontic patient. Am J Orthod Dentofacial Orthop 2006; 129: 431-435.

34. Gonçalves TS, Menezes LM, Silva LEA. Residual Monomer of Autopolymerized Acrylic Resin According to Different Manipulation and Polishing Methods. Angle Orthod 2008; 78:722-727.

35. Hagio M, Kawaguchi M, Motokawa W, Miyazaki K. Degradation of Methacrylate Monomers in Human Saliva. Dent Mater J 2006; 25: 241-246.

36. Huang FM, Tai KW, Hu CC, Chang YC. Cytotoxic effects of denture base materials on a permanent human oral epithelial cell line and on primary human oral fibroblasts in vitro. Int J Prosthodont 2001; 14:439-443.

37. Huang TH, Tsai CY, Chen SL, Kao CT. An evaluation of the cytotoxic effects of orthodontic bonding adhesives upon a primary human oral gingival fibroblast culture and a permanent, human oral cancer –cell line. J Biomed Mater Res (Appl Biomater) 2002; 63:814-821.

38. International Standard ISO Specification 7405: Dentistry - Evaluation of biocompatibility of medical devices used in Dentistry. International Organization for Standardization 2008. 3rd edition, Geneva, Switzerland

39. International Standard ISO Specification 10993-1: Biological evaluation of medical devices - Part 1: Evaluation and testing within a risk management process. International Organization for Standardization 2009, 3rd edition, Geneva, Switzerland.

40. International Standard ISO Specification 10993-5: Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity. International Organization for Standardization 2009, 3rd edition, Geneva, Switzerland.

41. International Standard ISO Specification 10993-12: Biological evaluation of medical devices - Part 12: Sample preparation and reference material. International Organization for Standardization 2007. 3rd edition, Geneva, Switzerland.

42. International Standard ISO Specification 20795-1: Dentistry- Base polymers-Part 1: Denture base polymers. International Organization for Standardization 2008. 1st edition, Geneva, Switzerland.

43. Ishikawa A, Jinno S, Suzuki T, Hayashi T, Kawai T, Mizuno T, Mori T, Hattori M. Global gene expression analyses of mouse fibroblast L929 cells exposed to IC50 MMA by DNA microarray and confirmation of four detoxification genes' expression by real-time PCR. Dent Mater J 2006; 25:205-213.

44. Issa Y, Watts DC, Brunton PA, Waters CM, Duxbury AJ. Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts in vitro. Dent Mater. 2004; 20:12-20.

45. Jaffer F, Finer Y, Santerre JP. Interactions between resin monomers and commercial composite resins with human saliva derived esterases. Biomaterials 2002; 23: 1707-1719.

46. Jorge JH, Giampaolo ET, Machado AL, Vergani CE. Cytotoxicity of denture base acrylic resins: a literature review. J Prosthet Dent 2003; 90: 190-193.

47. Jorge JH, Giampaolo ET, Vergani CE, Machado AL, Pavarina AC, Carlos IZ. Cytotoxicity of Denture Base Resins: effect of water bath and microwave postpolymerization heat treatments. Int J Prosthodont 2004; 17:340-344.

48. Jorge JH, Giampaolo ET, Vergani CE, Machado AL, Pavarina AC, Carlos IZ. Effect of post-polymerization heat treatments on the cytotoxicity of two denture base acrylic resins. J Appl Oral Sci 2006; 14:203-207.

49. Jorge JH, Giampaolo ET, Vergani CE, Machado Al, Pavarina AC, Carlos IZ. Biocompatibility of denture base acrylic resins evaluated in culture of L929 cells. Effect of polymerization cycle and post-polymerization treatments. Gerodontology 2007; 24:52-57.

50. Kedjarune U, Charoenworaluk N, Koontongkaew S. Release of methyl methacrylate from heat-cured and autopolymerized resins: cytotoxicity testing related to residual monomer. Aust Dent J 1999; 44: 25-30.

51. Koda T, Tsuchiya H, Yamauchi M, Hoshino Y, Takagi N, Kawano J. Highperformance liquid chromatograohic estimation of eluates from denture base polymers. J Dent 1989; 17:84-89.

52. Koda T, Tsuchiya H, Yamauchi M, Ohtani S, Takagi N, Kawano J. Leachability of denture-base acrylic resins in artificial saliva. Dent Mater 1990; 6:13-16.

53. Kournetas N. Impact of artificial ageing process on the wear resistance of dental materials. MA Thesis, Tübingen; 2005.

54. Lai YL, Chen YT, Lee SY, Shieh TM, Hung SL. Cytotoxic effects of dental resin liquids on primary gingival fibroblasts and periodontal ligament cells in vitro. J Oral Rehabil 2004; 31:1165-1172.

55. Larsen IB, Munksgaard EC. Effect of human saliva on surface degradation of composite resins. Scand J Dent Res 1991; 99:254-261.

56. Lee SY, Lai YL, Hsu TS. Influence of polymerization conditions on monomer elution and microhardness of autopolymerized polymethyl methacrylate resin. Eur J Oral Sci 2002; 110: 179–183.

57. Lefebvre CA, Knoernschild K, Schuster GS. Cytotoxicity of eluates from lightpolymerized denture base resins. J Prosthet Dent 1994; 72:644-650.

58. Lefebvre CA, Schuster GS, Marr JC, Knoernschild KL. The effect of pH on the cytotoxicity of eluates from denture Base resins. Int J Prosthodont 1995; 8:122-128.

59. Leggat PA, Kedjarune U. Toxicity of methylmethacrylate in dentistry. Int Dent J 2003; 53:126-131.

60. Lin BA, Jaffer F, Duff MD, Tang YW, Santerre JP. Identifying enzyme activities within human saliva which are relevant to dental resin composite biodegradation. Biomaterials 2005; 26:4259-4264.

61. Masuki K, Nomura Y, Bhawal UK, Sawajiri M, Hirata I, Nahara Y, Okazaki M. Apoptotic and necrotic influence of dental resin polymerization initiators in human gingival fibroblasts cultures. Dent Mater J 2007; 26:861-869.

62. Munksgaard EC, Freund M. Enzymatic hydrolysis of (di)methacrylates and their polymers. Scand J Dent Res 1990; 98:261-267

63. Munksgaard EC. Plasticizers in denture soft-lining materials: leaching and biodegradation. Eur J Oral Sci 2005; 113:166-169.

64. Minagi S, Tsunoda T, Yoshida K, Tsuru H. Objective testing of the efficiency of denture-cleansing agents. J Prosthet Dent 1987; 58:595-598.

65. Park JG, Ye Q, Topp EM, Lee CH, Kostoryz EL, Misra A, Spencer P. Dynamic Mechanical Analysis and Esterase Degradation of Dentin Adhesives Containing a Branched Methacrylate. J Biomed Mater Res Part B: Appl Biomater 2009; 91B: 61–70.

66. Rawls HR. Dental Polymers. In: Anusavice, K.J, editor. Phillips' Science of Dental Materials. St Louis: W. B. Saunder Company; 2003. p.143-169.

67. Ruyter IE. Release of formaldehyde from denture base polymers. Acta Odontol Scand 1980; 38; 17-27

68. Santerre JP, Shajii L, Leung BW. Relation of dental composite formulations to their degradation and the release of hydrolyzed polymeric-resin-derived products. Crit Rev Oral Biol Med 2001; 12:136-151.

69. Sato T, Takahashi H, Hongo T, Hayakawa I. Effect of degradation of denture base resin on bond strength to relining resins. Dent Mat J 2007; 26:89-95.

70. Schuster GS, Lefebvre CA, Dirksen TR, Knoernschild KL, Caughmann GB. Relationship between denture base resin cytotoxicity and cell lipid metabolism. Int J Prosthodont 1995; 8:580-586.

71. Seiss M, Track N, Hickel R, Reichl FX. In vitro stability of methylmethacrylic acid, TEGDMA and HEMA exposed to esterases. Dent Mater 2009; 25:1044-1049.

72. Sheridan PJ, Koka S, Ewoldsen NO, Lefebvre CA, Lavin MT. Cytotoxicity of denture base resins. Int J Prosthodont 1997; 10:73-77.

73. Takahashi JM, Machado FM, Nunez JM, Consani RL, Mesquita MF. Relining of prosthesis with auto-polymerizing hard denture reline resins: effect of post-polymerization treatment on flexural strength. Gerodontology. 2009; 26:232-236.

74. Tandon R, Gupta S, Agarwal SK. Denture base materials: From past to future. Indian Journal of Dental Sciences 2010; 2: 33-39

75. Tsuchiya H, Hoshino Y, Tajima K, Takagi N. Leaching and cytotoxicity of formaldehyde and methylmethacrylate from acrylic resin denture base materials. J Prosthet Dent 1994; 71:618-624.

76. Urban VM, Machado AL, Oliveira RV, Vergani CE, Pavarina AC, Cass QB. Residual monomer of reline acrylic resins. Effect of water-bath and microwave post-polymerization treatments. Dent Mater 2007; 23:363-368.

77. Urban VM, Machado AL, Vergani CE, Giampaolo ET, Pavarina AC, Almeida FG, Cass Q. Effect of water-bath polymerization on the mechanical properties, degree of conversion, and leaching of residual compounds of hard chairside reline resins. Dent Mater 2009; 25:662-671.

78. Vallittu PK, Ruyter IE, Buykuilmaz S. Effect of polymerization temperature and time on the residual monomer content of denture base polymers. Eur J Oral Sci 1998; 106: 588-593.

79. Yang H, Chou L, Chou M, Ghang Y. Assessment of genetic damage by methyl methacrylate employing in vitro mammalian test system. Biomaterials 2003; 24: 2909-2914.

80. Yoshii E. Cytotoxic effects of acrylates and methacrylates: Relationships of monomer structures and cytotoxicity. J Biomed Mater Res A 1997; 37:517-524.

81. Yourtee DM, Smith RE, Russo KA, Burmaster S, Cannon JM, Eick JD, Kostoryz EL. The stability of methacrylate biomaterials when enzyme challenged: kinetic and systematic evaluations. J Biomed Mat Res 2001; 57:522-531.

7. Appendix

7.1. List of Figures and Tables

		Page
Figure 1	Representation of the AChE-catalyzed hydrolysis reaction of the IBMA.	4
Figure 2	Representative image of 90-100% cell confluence. Phase contrast microscopy (x20) of human adult dermal fibroblasts.	5
Figure 3	a) Neubauer camera used for counting the cells on the microscope; b) Motic AE 2000 Binocular Inverted Microscope.	6
Figure 4	Laminar flow cabinet to work under sterile conditions.	7
Figure 5	a) Mixture and mould between polyester sheets and glass plates; b) Examples of two Probase Cold disk shaped specimens; c) Randomly division of two Probase Cold specimens; d) Pressure device Ivomat (IvoclarVivadent, Lichenstein).	8
Figure 6	UV specimens sterilization.	9
Figure 7	a) Motic AE 2000 Binocular Inverted Microscope; b) Representative image of subconfluent monolayer (x10).	11
Figure 8	a-c) Representative image of formazan crystals (x20, x40 and x100, respectively); d) Representative 96 well-plate before absorbance reading.	12
Figure 9	Effect of acetylcholinesterase on the cytotoxicity of 3 reline resins expressed as percentage of viable fibroblast present after exposure compared with the negative control group set as 100%; * means significant differences between experimental and control groups.	14
Figure 10	Cytotoxicity of Kooliner (K) and Ufigel Hard (U) dilutions expressed as percentage of viable fibroblasts present after exposure compared with the negative control group set as 100%.	15
Figure 11	Effect of acetylcholinesterase on the cytotoxicity of 3 reline resins	15

expressed as percentage of viable fibroblast present after exposure compared with the negative control group set as 100%.

Figure 12 Cellular viability as determined by MTT assay. Percentage of cellular viability of cells treated with increasing concentrations of HDMA for 24h. Results are expressed as the mean ± s.d. IC50 determination. 16

Figure 13 Cellular viability as determined by MTT assay. Percentage of cellular viability of cells treated with increasing concentrations of IBMA for 24h. Results are expressed as the mean ± s.d. IC50 determination. 16

Figure 14 Cellular viability as determined by MTT assay. Percentage of cellular viability of cells treated with increasing concentrations of MA for 24h. Results are expressed as the mean ± s.d. IC50 determination. 17

Figure 15 Analysis of curves and comparison of Ufi Gel Hard liquid and HDMA IC50. 18

Figure 16 Analysis of curves and comparison of Kooliner liquid and IBMA IC50. 18

Figure 17 Analysis of curves and comparison of Probase Cold liquid and MMA IC50. 18

Table 1 Materials under evaluation in the study. 7

7.2. List of Abbreviations

- ✓ **AChE:** Acetylcholinesterase
- ✓ **BMA:** Butyl methacrylate
- ✓ **C:** Control
- ✓ **Cm** Centimetre
- ✓ **DMEM:** Dulbecco's Modified Eagle's Medium
- ✓ **DMSO:** Dimethylsulfoxide
- ✓ **EDTA:** Ethylenediaminetetraacetic acid
- ✓ **FBS:** Fetal bovine serum
- ✓ **G:** G-force
- ✓ **H:** Hour
- ✓ **HEMA:** 2-hydroxyethyl methacrylate
- ✓ **HDMA:** 1.6- Hexanodioldimethacrylate
- ✓ **HPLC:** High-performance liquid chromatography
- ✓ **IBMA:** Isobutyl methacrylate
- ✓ **IC50:** Half maximal inhibitory concentration
- ✓ **ISO:** International Organization for Standardization
- ✓ **K:** Kooliner
- ✓ **KCl:** Potassium chloride
- ✓ **L:** Litre
- ✓ **LDH:** Lactate dehydrogenase assay
- ✓ **M:** Molar; Molarity; Molar concentration
- ✓ **MA:** Methacrylic acid
- ✓ **Mg:** Milligram
- ✓ **Min:** Minute

- ✓ **ML:** Millilitre
- ✓ **Mm:** Millimetre
- ✓ **MMA:** Methylmethacrylate
- ✓ **Mmol:** Millimole
- ✓ **MTT:** 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl-2*H*-tetrazolium bromide
- ✓ **NaCl** Sodium chloride
- ✓ **Nm:** Nanometre
- ✓ **PBS:** Phosphate buffered saline
- ✓ **PC:** Probase Cold
- ✓ **PEMA:** Polyethyl methacrylate
- ✓ **PMMA:** Polymethyl methacrylate
- ✓ **RM:** Residual monomer
- ✓ **U:** Ufi Gel Hard
- ✓ **UV:** Ultraviolet light
- ✓ **µL:** Microlitre

7.3. Experimental data

7.3.1. Cytotoxicity of the eluates

Experiment	Material	Group	Dilution (%)	Absorbance	Cell Viability (%)
1	K	AChE	50	0,17	52,4691
1	K	AChE	50	0,23	70,9877
1	K	AChE	50	0,211	65,1235
1	K	AChE	50	0,202	62,3457
1	K	Control	50	0,198	61,1111
1	K	Control	50	0,286	88,2716
1	K	Control	50	0,268	82,716
1	K	Control	50	0,195	60,1852
1	K	AChE	75	0,188	58,0247
1	K	AChE	75	0,209	64,5062
1	K	AChE	75	0,217	66,9753
1	K	AChE	75	0,212	65,4321
1	K	Control	75	0,261	80,5556
1	K	Control	75	0,298	91,9753
1	K	Control	75	0,195	60,1852
1	K	Control	75	0,197	60,8025
1	K	AChE	100	0,049	15,1235
1	K	AChE	100	0,045	13,8889
1	K	AChE	100	0,058	17,9012
1	K	AChE	100	0,044	13,5802
1	K	Control	100	0,068	20,9877
1	K	Control	100	0,062	19,1358
1	K	Control	100	0,044	13,5802
2	K	Control	50	0,235	47,5709
2	K	Control	50	0,245	49,5951
2	K	Control	50	0,274	55,4656
2	K	Control	50	0,217	43,9271
2	K	AChE	50	0,297	60,1215
2	K	AChE	50	0,29	58,7045
2	K	AChE	50	0,231	46,7611
2	K	AChE	50	0,216	43,7247
2	K	Control	75	0,072	14,5749
2	K	Control	75	0,092	18,6235
2	K	Control	75	0,128	25,9109
2	K	Control	75	0,124	25,1012
2	K	AChE	75	0,108	21,8623
2	K	AChE	75	0,113	22,8745
2	K	AChE	75	0,118	23,8866
2	K	AChE	75	0,079	15,9919

2	K	Control	100	0,045	9,1093
2	K	Control	100	0,042	8,502
2	K	Control	100	0,067	13,5628
2	K	Control	100	0,063	12,753
2	K	AChE	100	0,031	6,2753
2	K	AChE	100	0,033	6,6802
2	K	AChE	100	0,042	8,502
2	K	AChE	100	0,037	7,4899
3	K	AChE	50	0,2	58,8452
3	K	AChE	50	0,2	58,8452
3	K	AChE	50	0,183	53,8433
3	K	AChE	50	0,206	60,6105
3	K	Control	50	0,249	73,2622
3	K	Control	50	0,261	76,7929
3	K	Control	50	0,26	76,4987
3	K	Control	50	0,288	84,737
3	K	AChE	75	0,163	47,9588
3	K	AChE	75	0,157	46,1935
3	K	AChE	75	0,195	57,374
3	K	AChE	75	0,144	42,3685
3	K	Control	75	0,223	65,6124
3	K	Control	75	0,25	73,5565
3	K	Control	75	0,253	74,4391
3	K	Control	75	0,235	69,1431
3	K	AChE	100	0,091	26,7745
3	K	AChE	100	0,13	38,2494
3	K	AChE	100	0,095	27,9515
3	K	AChE	100	0,108	31,7764
4	K	Control	50	0,291	56,9472
4	K	Control	50	0,352	68,8845
4	K	Control	50	0,334	65,362
4	K	Control	50	0,387	75,7339
4	K	AChE	50	0,247	48,3366
4	K	AChE	50	0,26	50,8806
4	K	AChE	50	0,234	45,7926
4	K	AChE	50	0,275	53,816
4	K	Control	75	0,285	55,773
4	K	Control	75	0,281	54,9902
4	K	Control	75	0,303	59,2955
4	K	Control	75	0,28	54,7945
4	K	AChE	75	0,174	34,0509
4	K	AChE	75	0,177	34,638
4	K	AChE	75	0,167	32,681
4	K	AChE	75	0,139	27,2016
4	K	AChE	100	0,126	24,6575

4	K	AChE	100	0,117	22,8963
4	K	AChE	100	0,119	23,2877
4	K	AChE	100	0,124	24,2661
5	K	Control	50	0,2236	62,1746
5	K	Control	50	0,1739	48,3549
5	K	Control	50	0,1522	42,321
5	K	Control	50	0,165	45,8802
5	K	Control	50	0,1608	44,7123
5	K	Control	50	0,1549	43,0717
5	K	Control	50	0,1915	53,2488
5	K	Control	50	0,2077	57,7534
5	K	AChE	50	0,1765	55,7803
5	K	AChE	50	0,1902	60,11
5	K	AChE	50	0,2288	72,309
5	K	AChE	50	0,1935	61,1529
5	K	AChE	50	0,2185	69,0538
5	K	AChE	50	0,1949	61,5953
5	K	AChE	50	0,1682	53,1572
5	K	AChE	50	0,1759	55,5907
5	K	Control	75	0,0456	12,6796
5	K	Control	75	0,0317	8,8146
5	K	Control	75	0,028	7,7857
5	K	Control	75	0,0312	8,6755
5	K	Control	75	0,0282	7,8413
5	K	Control	75	0,0305	8,4809
5	K	Control	75	0,025	6,9515
5	K	Control	75	0,0371	10,3161
5	K	AChE	75	0,1588	50,1865
5	K	AChE	75	0,1509	47,6898
5	K	AChE	75	0,1498	47,3421
5	K	AChE	75	0,1457	46,0464
5	K	AChE	75	0,1563	49,3964
5	K	AChE	75	0,158	49,9336
5	K	AChE	75	0,1637	51,735
5	K	AChE	75	0,1507	47,6266
5	K	Control	100	0,0123	3,4202
5	K	Control	100	0,0133	3,6982
5	K	Control	100	0,0151	4,1987
5	K	Control	100	0,0134	3,726
5	K	Control	100	0,0151	4,1987
5	K	Control	100	0,014	3,8929
5	K	Control	100	0,0205	5,7003
5	K	Control	100	0,0168	4,6714
5	K	AChE	100	0,0986	31,1611
5	K	AChE	100	0,0986	31,1611

5	K	AChE	100	0,0686	21,68
5	K	AChE	100	0,0742	23,4498
5	K	AChE	100	0,0822	25,9781
5	K	AChE	100	0,0769	24,3031
5	K	AChE	100	0,0874	27,6215
5	K	AChE	100	0,0901	28,4748
6	K	Control	50	0,2497	76,6598
6	K	Control	50	0,2437	74,8177
6	K	Control	50	0,2316	71,1029
6	K	Control	50	0,2753	84,5191
6	K	Control	50	0,2116	77,495
6	K	Control	50	0,2138	78,3007
6	K	Control	50	0,2136	78,2274
6	K	Control	50	0,2274	83,2815
6	K	AChE	50	0,3104	95,2951
6	K	AChE	50	0,3213	98,6415
6	K	AChE	50	0,3187	97,8433
6	K	AChE	50	0,2994	91,918
6	K	AChE	50	0,2371	86,8339
6	K	AChE	50	0,2346	85,9183
6	K	AChE	50	0,2202	80,6446
6	K	AChE	50	0,2121	77,6781
6	K	Control	75	0,2093	64,2567
6	K	Control	75	0,2027	62,2304
6	K	Control	75	0,2059	63,2128
6	K	Control	75	0,1904	58,4542
6	K	Control	75	0,1726	63,2119
6	K	Control	75	0,1691	61,93
6	K	Control	75	0,1687	61,7836
6	K	Control	75	0,1669	61,1243
6	K	AChE	75	0,2084	63,9804
6	K	AChE	75	0,2096	64,3488
6	K	AChE	75	0,2129	65,3619
6	K	AChE	75	0,2119	65,0549
6	K	AChE	75	0,1942	71,1225
6	K	AChE	75	0,1707	62,516
6	K	AChE	75	0,1553	56,876
6	K	AChE	75	0,16	58,5973
7	K	Control	50	0,1265	52,2727
7	K	Control	50	0,1008	41,6529
7	K	Control	50	0,1107	45,7438
7	K	Control	50	0,1253	51,7769
7	K	Control	50	0,1304	54,7899
7	K	Control	50	0,0996	41,8487
7	K	Control	50	0,0538	22,605

7	K	Control	50	0,075	31,5126
7	K	AChE	50	0,1213	50,124
7	K	AChE	50	0,1213	50,124
7	K	AChE	50	0,121	50
7	K	AChE	50	0,1081	44,6694
7	K	AChE	50	0,1477	62,0588
7	K	AChE	50	0,1377	57,8571
7	K	AChE	50	0,1422	59,7479
7	K	AChE	50	0,1211	50,8824
7	K	Control	75	0,0273	11,281
7	K	Control	75	0,0222	9,1736
7	K	Control	75	0,0408	16,8595
7	K	Control	75	0,0269	11,1157
7	K	Control	75	0,0278	11,6807
7	K	Control	75	0,0203	8,5294
7	K	Control	75	0,0273	11,4706
7	K	Control	75	0,0207	8,6975
7	K	AChE	75	0,12	49,5868
7	K	AChE	75	0,1275	52,686
7	K	AChE	75	0,1019	42,1074
7	K	AChE	75	0,1108	45,7851
7	K	AChE	75	0,0491	20,6303
7	K	AChE	75	0,0287	12,0588
7	K	AChE	75	0,0283	11,8908
7	K	AChE	75	0,0227	9,5378
7	K	Control	100	0,0224	9,2562
7	K	Control	100	0,0196	8,0992
7	K	Control	100	0,0265	10,9504
7	K	Control	100	0,0231	9,5455
7	K	Control	100	0,0235	9,8739
7	K	Control	100	0,0332	13,9496
7	K	Control	100	0,0174	7,3109
7	K	Control	100	0,0184	7,7311
7	K	AChE	100	0,0239	9,876
7	K	AChE	100	0,0309	12,7686
7	K	AChE	100	0,0259	10,7025
7	K	AChE	100	0,0271	11,1983
7	K	AChE	100	0,017	7,1429
7	K	AChE	100	0,0217	9,1176
7	K	AChE	100	0,0227	9,5378
7	K	AChE	100	0,022	9,2437
1	U	Control	50	0,337	104,0123
1	U	AChE	50	0,363	112,037
1	U	AChE	50	0,396	122,2222
1	U	Control	75	0,253	78,0864

1	U	Control	75	0,244	75,3086
1	U	Control	75	0,235	72,5309
1	U	Control	75	0,228	70,3704
1	U	AChE	75	0,255	78,7037
1	U	AChE	75	0,36	111,1111
1	U	AChE	75	0,291	89,8148
1	U	Control	100	0,151	46,6049
1	U	AChE	100	0,288	88,8889
2	U	Control	50	0,435	88,0567
2	U	Control	50	0,371	75,1012
2	U	AChE	50	0,611	123,6842
2	U	AChE	50	0,557	112,753
2	U	AChE	50	0,552	111,7409
2	U	AChE	50	0,436	88,2591
2	U	Control	75	0,194	39,2713
2	U	Control	75	0,295	59,7166
2	U	Control	75	0,261	52,834
2	U	Control	75	0,243	49,1903
2	U	AChE	75	0,332	67,2065
2	U	AChE	75	0,396	80,1619
2	U	AChE	75	0,396	80,1619
2	U	AChE	75	0,286	57,8947
2	U	Control	100	0,172	34,8178
2	U	Control	100	0,173	35,0202
2	U	Control	100	0,16	32,3887
2	U	AChE	100	0,279	56,4777
2	U	AChE	100	0,355	71,8623
2	U	AChE	100	0,287	58,0972
2	U	AChE	100	0,345	69,8381
3	U	Control	50	0,34	100,0368
3	U	Control	50	0,352	103,5675
3	U	Control	50	0,379	111,5116
3	U	Control	50	0,309	90,9158
3	U	AChE	50	0,317	93,2696
3	U	AChE	50	0,346	101,8021
3	U	AChE	50	0,374	110,0405
3	U	AChE	50	0,373	109,7462
3	U	Control	75	0,355	104,4502
3	U	Control	75	0,341	100,331
3	U	Control	75	0,328	96,5061
3	U	Control	75	0,296	87,0908
3	U	AChE	75	0,367	107,9809
3	U	AChE	75	0,3	88,2677
3	U	AChE	75	0,349	102,6848
3	U	AChE	75	0,341	100,331

3	U	Control	100	0,145	42,6627
3	U	Control	100	0,181	53,2549
3	U	Control	100	0,111	32,6591
3	U	AChE	100	0,314	92,3869
3	U	AChE	100	0,268	78,8525
3	U	AChE	100	0,281	82,6775
3	U	AChE	100	0,261	76,7929
4	U	Control	50	0,489	95,6947
4	U	Control	50	0,52	101,7613
4	U	Control	50	0,522	102,1526
4	U	Control	50	0,476	93,1507
4	U	AChE	50	0,451	88,2583
4	U	AChE	50	0,532	104,1096
4	U	AChE	50	0,581	113,6986
4	U	AChE	50	0,548	107,2407
4	U	Control	75	0,401	78,4736
4	U	Control	75	0,477	93,3464
4	U	Control	75	0,457	89,4325
4	U	Control	75	0,474	92,7593
4	U	AChE	75	0,451	88,2583
4	U	AChE	75	0,462	90,411
4	U	AChE	75	0,456	89,2368
4	U	AChE	75	0,427	83,5616
4	U	AChE	100	0,424	82,9746
4	U	AChE	100	0,422	82,5832
4	U	AChE	100	0,39	76,3209
4	U	AChE	100	0,444	86,8885
5	U	Control	50	0,3332	92,6501
5	U	Control	50	0,3458	96,1537
5	U	Control	50	0,3558	98,9343
5	U	Control	50	0,3682	102,3823
5	U	Control	50	0,3793	105,4688
5	U	Control	50	0,3473	96,5708
5	U	Control	50	0,358	99,5461
5	U	Control	50	0,3539	98,406
5	U	AChE	50	0,3111	98,3187
5	U	AChE	50	0,2943	93,0093
5	U	AChE	50	0,2799	88,4584
5	U	AChE	50	0,2724	86,0881
5	U	AChE	50	0,2905	91,8084
5	U	AChE	50	0,2784	87,9843
5	U	AChE	50	0,3071	97,0545
5	U	AChE	50	0,2807	88,7112
5	U	Control	75	0,2699	75,0488
5	U	Control	75	0,2308	64,1766

5	U	Control	75	0,3692	102,6604
5	U	Control	75	0,326	90,6481
5	U	Control	75	0,3496	97,2103
5	U	Control	75	0,3697	102,7994
5	U	Control	75	0,2898	80,5823
5	U	Control	75	0,3594	99,9354
5	U	AChE	75	0,2508	79,2617
5	U	AChE	75	0,2686	84,8872
5	U	AChE	75	0,2752	86,973
5	U	AChE	75	0,2604	82,2957
5	U	AChE	75	0,2439	77,0811
5	U	AChE	75	0,2799	88,4584
5	U	AChE	75	0,237	74,9004
5	U	AChE	75	0,2153	68,0425
5	U	Control	100	0,2308	64,1766
5	U	Control	100	0,2536	70,5164
5	U	Control	100	0,2364	65,7338
5	U	AChE	100	0,2369	74,8688
5	U	AChE	100	0,2234	70,6024
5	U	AChE	100	0,2085	65,8934
5	U	AChE	100	0,2098	66,3043
5	U	AChE	100	0,2033	64,25
5	U	AChE	100	0,216	68,2637
5	U	AChE	100	0,1909	60,3312
5	U	AChE	100	0,1884	59,5411
6	U	Control	50	0,3717	114,1147
6	U	Control	50	0,3724	114,3296
6	U	Control	50	0,3537	108,5885
6	U	Control	50	0,3406	104,5667
6	U	Control	50	0,2996	109,7235
6	U	Control	50	0,2962	108,4783
6	U	Control	50	0,3022	110,6757
6	U	Control	50	0,2894	105,9879
6	U	AChE	50	0,2775	85,1946
6	U	AChE	50	0,2786	85,5323
6	U	AChE	50	0,276	84,7341
6	U	AChE	50	0,2569	78,8702
6	U	AChE	50	0,2297	84,1238
6	U	AChE	50	0,2407	88,1524
6	U	AChE	50	0,2501	91,5949
6	U	AChE	50	0,2323	85,076
6	U	Control	75	0,3616	111,0139
6	U	Control	75	0,3751	115,1585
6	U	Control	75	0,3602	110,5841
6	U	Control	75	0,3393	104,1676

6	U	Control	75	0,2849	104,3399
6	U	Control	75	0,3076	112,6534
6	U	Control	75	0,2905	106,3908
6	U	Control	75	0,2765	101,2635
6	U	AChE	75	0,2688	82,5236
6	U	AChE	75	0,2646	81,2342
6	U	AChE	75	0,2575	79,0544
6	U	AChE	75	0,2204	67,6644
6	U	AChE	75	0,2146	78,5937
6	U	AChE	75	0,2296	84,0872
6	U	AChE	75	0,2091	76,5794
6	U	AChE	75	0,2215	81,1207
6	U	Control	100	0,2251	69,1074
6	U	Control	100	0,2342	71,9011
6	U	Control	100	0,2104	64,5944
6	U	AChE	100	0,1958	60,1121
6	U	AChE	100	0,2014	61,8313
6	U	AChE	100	0,1853	56,8885
6	U	AChE	100	0,1973	60,5726
6	U	AChE	100	0,1895	69,4012
6	U	AChE	100	0,1717	62,8823
6	U	AChE	100	0,1804	66,0685
6	U	AChE	100	0,1758	64,3838
7	U	Control	50	0,1681	69,4628
7	U	Control	50	0,2383	98,4711
7	U	Control	50	0,181	74,7934
7	U	Control	50	0,1983	81,9421
7	U	Control	50	0,2157	90,6303
7	U	Control	50	0,2114	88,8235
7	U	Control	50	0,1961	82,395
7	U	Control	50	0,1998	83,9496
7	U	AChE	50	0,199	82,2314
7	U	AChE	50	0,2247	92,8512
7	U	AChE	50	0,2082	86,0331
7	U	AChE	50	0,1544	63,8017
7	U	AChE	50	0,2402	100,9244
7	U	AChE	50	0,2135	89,7059
7	U	AChE	50	0,2397	100,7143
7	U	AChE	50	0,2353	98,8655
7	U	Control	75	0,1538	63,5537
7	U	Control	75	0,1488	61,4876
7	U	Control	75	0,1726	71,3223
7	U	Control	75	0,1784	73,719
7	U	Control	75	0,2067	86,8487
7	U	Control	75	0,175	73,5294

7	U	Control	75	0,1921	80,7143
7	U	Control	75	0,1676	70,4202
7	U	AChE	75	0,1635	67,562
7	U	AChE	75	0,2398	99,0909
7	U	AChE	75	0,1809	74,7521
7	U	AChE	75	0,1833	75,7438
7	U	AChE	75	0,2315	97,2689
7	U	AChE	75	0,2754	115,7143
7	U	AChE	75	0,2423	101,8067
7	U	Control	100	0,0789	32,6033
7	U	Control	100	0,0884	36,5289
7	U	Control	100	0,0807	33,9076
7	U	Control	100	0,0819	34,4118
7	U	AChE	100	0,2368	97,8512
7	U	AChE	100	0,2444	100,9917
7	U	AChE	100	0,1874	77,438
7	U	AChE	100	0,1892	78,1818
1	P	Control	50	0,386	119,1358
1	P	Control	50	0,382	117,9012
1	P	Control	50	0,418	129,0123
1	P	AChE	75	0,447	137,963
1	P	AChE	75	0,259	79,9383
1	P	Control	75	0,39	120,3704
1	P	Control	75	0,401	123,7654
1	P	Control	75	0,442	136,4198
1	P	Control	75	0,411	126,8519
1	P	AChE	100	0,387	119,4444
2	P	Control	50	0,413	83,6032
2	P	Control	50	0,495	100,2024
2	P	Control	50	0,622	125,9109
2	P	Control	50	0,582	117,8138
2	P	AChE	50	0,452	91,498
2	P	AChE	50	0,491	99,3927
2	P	AChE	50	0,455	92,1053
2	P	AChE	50	0,447	90,4858
2	P	Control	75	0,574	116,1943
2	P	Control	75	0,48	97,166
2	P	Control	75	0,545	110,3239
2	P	Control	75	0,569	115,1822
2	P	AChE	75	0,609	123,2794
2	P	AChE	75	0,598	121,0526
2	P	AChE	75	0,651	131,7814
2	P	AChE	75	0,573	115,9919
2	P	Control	100	0,424	85,83
2	P	Control	100	0,581	117,6113

2	P	AChE	100	0,394	79,7571
2	P	AChE	100	0,516	104,4534
2	P	AChE	100	0,376	76,1134
3	P	AChE	50	0,321	94,4465
3	P	AChE	50	0,369	108,5693
3	P	AChE	50	0,343	100,9195
3	P	AChE	50	0,305	89,7389
3	P	Control	50	0,259	76,2045
3	P	Control	50	0,305	89,7389
3	P	Control	50	0,286	84,1486
3	P	Control	50	0,389	114,4538
3	P	AChE	75	0,359	105,6271
3	P	AChE	75	0,349	102,6848
3	P	AChE	75	0,328	96,5061
3	P	AChE	75	0,317	93,2696
3	P	Control	75	0,296	87,0908
3	P	Control	75	0,304	89,4446
3	P	Control	75	0,305	89,7389
3	P	Control	75	0,379	111,5116
3	P	AChE	100	0,315	92,6811
3	P	AChE	100	0,292	85,9139
3	P	AChE	100	0,328	96,5061
3	P	AChE	100	0,326	95,9176
3	P	Control	100	0,343	100,9195
3	P	Control	100	0,325	95,6234
3	P	Control	100	0,329	96,8003
3	P	Control	100	0,296	87,0908
4	P	Control	50	0,602	117,8082
4	P	Control	50	0,513	100,3914
4	P	Control	50	0,472	92,3679
4	P	Control	50	0,596	116,6341
4	P	AChE	50	0,461	90,2153
4	P	AChE	50	0,437	85,5186
4	P	AChE	50	0,522	102,1526
4	P	AChE	50	0,458	89,6282
4	P	Control	75	0,517	101,1742
4	P	Control	75	0,49	95,8904
4	P	Control	75	0,572	111,9374
4	P	Control	75	0,61	119,3738
4	P	AChE	75	0,487	95,3033
4	P	AChE	75	0,535	104,6967
4	P	AChE	75	0,504	98,6301
4	P	AChE	75	0,487	95,3033
4	P	Control	100	0,492	96,2818
4	P	Control	100	0,598	117,0254

4	P	Control	100	0,471	92,1722
4	P	Control	100	0,531	103,9139
4	P	AChE	100	0,455	89,0411
4	P	AChE	100	0,419	81,9961
4	P	AChE	100	0,526	102,9354
4	P	AChE	100	0,402	78,6693
5	P	Control	50	0,3684	102,4379
5	P	Control	50	0,4236	117,7869
5	P	Control	50	0,3725	103,578
5	P	Control	50	0,3768	104,7736
5	P	Control	50	0,4097	113,9219
5	P	Control	50	0,333	92,5945
5	P	Control	50	0,3654	101,6037
5	P	Control	50	0,3012	83,7522
5	P	AChE	50	0,312	98,6031
5	P	AChE	50	0,318	100,4993
5	P	AChE	50	0,3413	107,863
5	P	AChE	50	0,3292	104,0389
5	P	AChE	50	0,3175	100,3413
5	P	AChE	50	0,3231	102,1111
5	P	AChE	50	0,3177	100,4045
5	P	AChE	50	0,3187	100,7206
5	P	Control	75	0,4066	113,0599
5	P	Control	75	0,3981	110,6963
5	P	Control	75	0,4053	112,6984
5	P	Control	75	0,3766	104,718
5	P	Control	75	0,3975	110,5295
5	P	Control	75	0,3646	101,3813
5	P	Control	75	0,3864	107,443
5	P	Control	75	0,377	104,8292
5	P	AChE	75	0,2976	94,0522
5	P	AChE	75	0,2906	91,84
5	P	AChE	75	0,3167	100,0885
5	P	AChE	75	0,3033	95,8536
5	P	AChE	75	0,297	93,8626
5	P	AChE	75	0,3034	95,8852
5	P	AChE	75	0,304	96,0748
5	P	AChE	75	0,2651	83,7811
5	P	Control	100	0,3742	104,0507
5	P	Control	100	0,3708	103,1053
5	P	Control	100	0,3739	103,9672
5	P	Control	100	0,3423	95,1805
5	P	Control	100	0,3248	90,3144
5	P	Control	100	0,346	96,2093
5	P	Control	100	0,294	81,7501

5	P	Control	100	0,362	100,6583
5	P	AChE	100	0,2859	90,3546
5	P	AChE	100	0,2659	84,0339
5	P	AChE	100	0,2844	89,8805
5	P	AChE	100	0,2921	92,314
5	P	AChE	100	0,2782	87,9211
5	P	AChE	100	0,3035	95,9168
5	P	AChE	100	0,2449	77,3971
5	P	AChE	100	0,2386	75,4061
6	P	Control	50	0,3259	100,0537
6	P	Control	50	0,3291	101,0362
6	P	Control	50	0,3649	112,027
6	P	Control	50	0,339	104,0755
6	P	Control	50	0,2874	105,2554
6	P	Control	50	0,263	96,3194
6	P	Control	50	0,2418	88,5552
6	P	Control	50	0,2693	98,6266
6	P	AChE	50	0,3822	117,3382
6	P	AChE	50	0,3501	107,4833
6	P	AChE	50	0,2692	98,59
6	P	AChE	50	0,2732	100,0549
6	P	AChE	50	0,2798	102,4721
6	P	AChE	50	0,2804	102,6918
6	P	Control	75	0,3245	99,6239
6	P	Control	75	0,2891	88,7559
6	P	Control	75	0,3478	106,7772
6	P	Control	75	0,3355	103,001
6	P	Control	75	0,2672	97,8575
6	P	Control	75	0,2414	88,4087
6	P	Control	75	0,2317	84,8563
6	P	Control	75	0,2441	89,3975
6	P	AChE	75	0,4036	123,9082
6	P	AChE	75	0,4006	122,9872
6	P	AChE	75	0,3776	115,926
6	P	AChE	75	0,3991	122,5267
6	P	AChE	75	0,3295	120,6739
6	P	AChE	75	0,3026	110,8222
6	P	AChE	75	0,2856	104,5962
6	P	AChE	75	0,3138	114,924
6	P	Control	100	0,3321	101,9572
6	P	Control	100	0,3674	112,7945
6	P	Control	100	0,3774	115,8646
6	P	Control	100	0,3661	112,3954
6	P	Control	100	0,2498	91,4851
6	P	Control	100	0,282	103,2778

6	P	Control	100	0,2887	105,7316
6	P	Control	100	0,2976	108,991
6	P	AChE	100	0,4025	123,5705
6	P	AChE	100	0,3745	114,9743
6	P	AChE	100	0,3102	113,6056
6	P	AChE	100	0,2938	107,5993
6	P	AChE	100	0,2702	98,9562
6	P	AChE	100	0,3326	121,8092
7	P	Control	50	0,2302	95,124
7	P	Control	50	0,2518	104,0496
7	P	Control	50	0,2416	99,8347
7	P	Control	50	0,2771	114,5041
7	P	Control	50	0,2367	99,4538
7	P	Control	50	0,2604	109,4118
7	P	Control	50	0,2694	113,1933
7	P	Control	50	0,2974	124,958
7	P	AChE	50	0,2522	104,2149
7	P	AChE	50	0,2282	94,2975
7	P	AChE	50	0,2254	93,1405
7	P	AChE	50	0,2279	94,1736
7	P	AChE	50	0,2657	111,6387
7	P	AChE	50	0,2389	100,3782
7	P	AChE	50	0,245	102,9412
7	P	Control	75	0,2363	97,6446
7	P	Control	75	0,2687	111,0331
7	P	Control	75	0,2797	115,5785
7	P	Control	75	0,2705	111,7769
7	P	Control	75	0,2635	110,7143
7	P	Control	75	0,2912	122,3529
7	P	Control	75	0,2753	115,6723
7	P	Control	75	0,292	122,6891
7	P	AChE	75	0,2749	113,595
7	P	AChE	75	0,265	109,5041
7	P	AChE	75	0,2809	116,0744
7	P	AChE	75	0,2243	92,686
7	P	AChE	75	0,3129	131,4706
7	P	AChE	75	0,2881	121,0504
7	P	AChE	75	0,2396	100,6723
7	P	AChE	75	0,2224	93,4454
7	P	Control	100	0,2666	110,1653
7	P	Control	100	0,2893	119,5455
7	P	Control	100	0,3018	124,7107
7	P	Control	100	0,3075	127,0661
7	P	Control	100	0,2564	107,7311
7	P	AChE	100	0,2868	118,5124

7	P	AChE	100	0,2871	118,6364
7	P	AChE	100	0,2437	100,7025
7	P	AChE	100	0,2348	97,0248
7	P	AChE	100	0,2989	125,5882
7	P	AChE	100	0,2819	118,4454
7	P	AChE	100	0,2589	108,7815
7	P	AChE	100	0,2147	90,2101

7.3.2. Cytotoxicity of the test compounds

Experiment	Compound	Concentration (mM)	Absorbance	Cell Viability (%)
1	HDMA	2,9	0,036	8,9441
1	HDMA	2,9	0,046	11,4286
1	HDMA	2,9	0,038	9,441
1	HDMA	2,9	0,05	12,4224
1	HDMA	2,9	0,044	10,9317
1	HDMA	2,9	0,049	12,1739
1	HDMA	1,5	0,066	16,3975
1	HDMA	1,5	0,062	15,4037
1	HDMA	1,5	0,048	11,9255
1	HDMA	1,5	0,04	9,9379
1	HDMA	1,5	0,056	13,913
1	HDMA	1,5	0,044	10,9317
1	HDMA	0,75	0,06	14,9068
1	HDMA	0,75	0,05	12,4224
1	HDMA	0,75	0,051	12,6708
1	HDMA	0,75	0,082	20,3727
1	HDMA	0,75	0,091	22,6087
1	HDMA	0,75	0,072	17,8882
1	HDMA	0,5	0,056	13,913
1	HDMA	0,5	0,081	20,1242
1	HDMA	0,5	0,086	21,3665
1	HDMA	0,5	0,096	23,8509
1	HDMA	0,5	0,086	21,3665
1	HDMA	0,5	0,061	15,1553
1	HDMA	0,38	0,099	24,5963
1	HDMA	0,38	0,181	44,9689
1	HDMA	0,38	0,128	31,8012
1	HDMA	0,38	0,127	31,5528
1	HDMA	0,38	0,142	35,2795
1	HDMA	0,38	0,069	17,1429
1	HDMA	0,3	0,09	30
1	HDMA	0,3	0,111	37

1	HDMA	0,3	0,102	34
1	HDMA	0,3	0,108	36
1	HDMA	0,3	0,111	37
1	HDMA	0,3	0,093	31
1	HDMA	0,1	0,454	112,795
1	HDMA	0,1	0,4	99,3789
1	HDMA	0,1	0,405	100,6211
1	HDMA	0,1	0,321	79,7516
1	HDMA	0,1	0,381	94,6584
1	HDMA	0,1	0,454	112,795
1	HDMA	0,05	0,42	104,3478
1	HDMA	0,05	0,368	91,4286
1	HDMA	0,05	0,477	118,5093
1	HDMA	0,05	0,456	113,2919
1	HDMA	0,05	0,484	120,2484
1	HDMA	0,05	0,486	120,7453
2	HDMA	0,2	0,176	58,6667
2	HDMA	0,2	0,225	75
2	HDMA	0,2	0,233	77,6667
2	HDMA	0,2	0,147	49
2	HDMA	0,15	0,277	92,3333
2	HDMA	0,15	0,289	96,3333
2	HDMA	0,15	0,311	103,6667
2	HDMA	0,15	0,215	71,6667
3	IBMA	32	0,035	9,1583
3	IBMA	32	0,029	7,5883
3	IBMA	32	0,031	8,1116
3	IBMA	32	0,028	7,3266
3	IBMA	32	0,035	9,1583
3	IBMA	32	0,028	7,3266
3	IBMA	20	0,025	6,5416
3	IBMA	20	0,03	7,85
3	IBMA	20	0,03	7,85
3	IBMA	20	0,032	8,3733
3	IBMA	20	0,034	8,8966
3	IBMA	20	0,03	7,85
3	IBMA	12,8	0,029	7,5883
3	IBMA	12,8	0,029	7,5883
3	IBMA	12,8	0,031	8,1116
3	IBMA	12,8	0,034	8,8966
3	IBMA	12,8	0,033	8,635
3	IBMA	12,8	0,032	8,3733
3	IBMA	10	0,032	8,3733
3	IBMA	10	0,042	10,99
3	IBMA	10	0,039	10,205

3	IBMA	10	0,032	8,3733
3	IBMA	10	0,033	8,635
3	IBMA	10	0,032	8,3733
3	IBMA	8	0,029	7,5883
3	IBMA	8	0,048	12,56
3	IBMA	8	-0,003	-0,785
3	IBMA	8	0,037	9,6816
3	IBMA	8	0,032	8,3733
3	IBMA	8	0,039	10,205
3	IBMA	6,4	0,029	7,5883
3	IBMA	6,4	0,036	9,42
3	IBMA	6,4	0,04	10,4666
3	IBMA	6,4	0,036	9,42
3	IBMA	6,4	0,107	27,9983
3	IBMA	6,4	0,207	54,1648
3	IBMA	3,2	0,165	43,1749
3	IBMA	3,2	0,193	50,5015
3	IBMA	3,2	0,209	54,6882
3	IBMA	3,2	0,098	25,6433
3	IBMA	3,2	0,26	68,0331
3	IBMA	3,2	0,347	90,7981
3	IBMA	1,5	0,386	101,0031
3	IBMA	1,5	0,314	82,1631
3	IBMA	1,5	0,362	94,7231
3	IBMA	1,5	0,367	96,0314
3	IBMA	1,5	0,383	100,2181
3	IBMA	1,5	0,367	96,0314
4	IBMA	1	0,476	89,5298
4	IBMA	1	0,502	94,4201
4	IBMA	1	0,567	106,6458
4	IBMA	1	0,598	112,4765
4	IBMA	1	0,525	98,7461
4	IBMA	1	0,572	107,5862
4	IBMA	0,5	0,57	107,21
4	IBMA	0,5	0,545	102,5078
4	IBMA	0,5	0,536	100,815
4	IBMA	0,5	0,585	110,0313
4	IBMA	0,5	0,564	106,0815
4	IBMA	0,5	0,598	112,4765
5	MMA	9	0,405	97,3558
5	MMA	9	0,414	99,5192
5	MMA	9	0,359	86,2981
5	MMA	9	0,48	115,3846
5	MMA	9	0,394	94,7115
5	MMA	9	0,493	118,5096

5	MMA	7,5	0,474	113,9423
5	MMA	7,5	0,351	84,375
5	MMA	7,5	0,344	82,6923
5	MMA	7,5	0,369	88,7019
5	MMA	7,5	0,343	82,4519
5	MMA	7,5	0,418	100,4808
5	MMA	5	0,405	97,3558
5	MMA	5	0,429	103,125
5	MMA	5	0,403	96,875
5	MMA	5	0,411	98,7981
5	MMA	5	0,428	102,8846
5	MMA	5	0,401	96,3942
5	MMA	4	0,363	87,2596
5	MMA	4	0,406	97,5962
5	MMA	4	0,333	80,0481
5	MMA	4	0,368	88,4615
5	MMA	4	0,317	76,2019
5	MMA	4	0,429	103,125
5	MMA	2	0,441	106,0096
5	MMA	2	0,423	101,6827
5	MMA	2	0,398	95,6731
5	MMA	2	0,326	78,3654
5	MMA	2	0,46	110,5769
5	MMA	2	0,388	93,2692
5	MMA	1	0,398	95,6731
5	MMA	1	0,364	87,5
5	MMA	1	0,475	114,1827
5	MMA	1	0,463	111,2981
5	MMA	1	0,452	108,6538
5	MMA	1	0,403	96,875
5	MMA	0,5	0,444	106,7308
5	MMA	0,5	0,355	85,3365
5	MMA	0,5	0,454	109,1346
5	MMA	0,5	0,402	96,6346
5	MMA	0,5	0,41	98,5577
5	MMA	0,5	0,494	118,75
5	MMA	0,1	0,352	84,6154
5	MMA	0,1	0,476	114,4231
5	MMA	0,1	0,444	106,7308
5	MMA	0,1	0,43	103,3654
5	MMA	0,1	0,413	99,2788
5	MMA	0,1	0,493	118,5096
6	MA	108,6	0,027	7,5093
6	MA	108,6	0,026	7,2311
6	MA	108,6	0,028	7,7874

6	MA	108,6	0,024	6,6749
6	MA	108,6	0,032	8,8999
6	MA	108,6	0,041	11,403
6	MA	36,2	0,035	9,7342
6	MA	36,2	0,022	6,1187
6	MA	36,2	0,023	6,3968
6	MA	36,2	0,032	8,8999
6	MA	36,2	0,028	7,7874
6	MA	36,2	0,033	9,178
6	MA	18,1	0,398	110,6922
6	MA	18,1	0,289	80,377
6	MA	18,1	0,369	102,6267
6	MA	18,1	0,29	80,6551
6	MA	9,05	0,385	107,0766
6	MA	9,05	0,391	108,7454
6	MA	9,05	0,335	93,1706
6	MA	9,05	0,406	112,9172
6	MA	9,05	0,341	94,8393
6	MA	9,05	0,381	105,9642
6	MA	3,62	0,411	114,3078
6	MA	3,62	0,419	116,5328
6	MA	3,62	0,322	89,555
6	MA	3,62	0,398	110,6922
6	MA	3,62	0,414	115,1422
6	MA	3,62	0,408	113,4734
6	MA	1,81	0,342	95,1174
6	MA	1,81	0,372	103,4611
6	MA	1,81	0,372	103,4611
6	MA	1,81	0,409	113,7515
6	MA	1,81	0,424	117,9234
6	MA	1,81	0,429	119,314
7	MA	30	0,299	71,875
7	MA	30	0,306	73,5577
7	MA	30	0,309	74,2788
7	MA	30	0,356	85,5769
7	MA	30	0,313	75,2404
7	MA	24	0,374	89,9038
7	MA	24	0,311	74,7596
7	MA	24	0,333	80,0481
7	MA	24	0,398	95,6731

7.3.3. Cytotoxicity of the acrylic reline resin liquids

Experiment	Reline Liquid	Concentration (mM)	Absorbance	Cell Viability (%)
1	Kooliner	30	0,023	5,612798265
1	Kooliner	30	0,026	6,344902386
1	Kooliner	30	0,027	6,588937093
1	Kooliner	30	0,027	6,588937093
1	Kooliner	30	0,028	6,8329718
1	Kooliner	30	0,025	6,100867679
1	Kooliner	18	0,03	7,321041215
1	Kooliner	18	0,025	6,100867679
1	Kooliner	18	0,028	6,8329718
1	Kooliner	18	0,026	6,344902386
1	Kooliner	18	0,028	6,8329718
1	Kooliner	18	0,025	6,100867679
1	Kooliner	12	0,026	6,344902386
1	Kooliner	12	0,026	6,344902386
1	Kooliner	12	0,034	8,297180043
1	Kooliner	12	0,03	7,321041215
1	Kooliner	12	0,035	8,541214751
1	Kooliner	12	0,036	8,785249458
3	Kooliner	9	0,022	6,921866807
3	Kooliner	9	0,044	13,84373361
3	Kooliner	9	0,037	11,64132145
3	Kooliner	9	0,025	7,865757735
3	Kooliner	9	0,033	10,38280021
3	Kooliner	9	0,033	10,38280021
1	Kooliner	6	0,283	69,06182213
1	Kooliner	6	0,248	60,52060738
1	Kooliner	6	0,258	62,96095445
1	Kooliner	6	0,234	57,10412148
1	Kooliner	3	0,414	101,0303688
1	Kooliner	3	0,413	100,7863341
1	Kooliner	3	0,415	101,2744035
1	Kooliner	3	0,379	92,48915401
1	Kooliner	3	0,401	97,85791757
1	Kooliner	3	0,399	97,36984816
1	Kooliner	1.5	0,455	111,0357918
1	Kooliner	1.5	0,423	103,2266811
1	Kooliner	1.5	0,362	88,34056399
1	Kooliner	1.5	0,392	95,66160521
2	Probase Cold	188	0,043	12,6223092
2	Probase Cold	188	0,029	8,512720157

2	Probase Cold	188	0,035	10,2739726
2	Probase Cold	188	0,033	9,686888454
2	Probase Cold	188	0,032	9,39334638
2	Probase Cold	188	0,039	11,4481409
2	Probase Cold	94	0,034	9,980430528
2	Probase Cold	94	0,038	11,15459883
2	Probase Cold	94	0,04	11,74168297
2	Probase Cold	94	0,025	7,338551859
2	Probase Cold	94	0,028	8,219178082
2	Probase Cold	94	0,042	12,32876712
2	Probase Cold	70.5	0,031	9,099804305
2	Probase Cold	70.5	0,039	11,4481409
2	Probase Cold	70.5	0,033	9,686888454
2	Probase Cold	70.5	0,043	12,6223092
2	Probase Cold	70.5	0,048	14,09001957
2	Probase Cold	70.5	0,034	9,980430528
2	Probase Cold	47	0,046	13,50293542
2	Probase Cold	47	0,041	12,03522505
2	Probase Cold	47	0,036	10,56751468
2	Probase Cold	47	0,045	13,20939335
2	Probase Cold	47	0,037	10,86105675
2	Probase Cold	47	0,046	13,50293542
2	Probase Cold	23.5	0,045	13,20939335
2	Probase Cold	23.5	0,042	12,32876712
2	Probase Cold	23.5	0,039	11,4481409
2	Probase Cold	23.5	0,039	11,4481409
2	Probase Cold	23.5	0,047	13,7964775
2	Probase Cold	23.5	0,045	13,20939335
2	Probase Cold	9.4	0,096	28,18003914
2	Probase Cold	9.4	0,111	32,58317025
2	Probase Cold	9.4	0,117	34,3444227
2	Probase Cold	9.4	0,067	19,66731898
2	Probase Cold	9.4	0,092	27,00587084
2	Probase Cold	9.4	0,063	18,49315068
3	Probase Cold	4.7	0,278	87,46722601
3	Probase Cold	4.7	0,239	75,19664394
3	Probase Cold	4.7	0,246	77,39905611
3	Probase Cold	4.7	0,28	88,09648663
3	Probase Cold	4.7	0,234	73,6234924
3	Probase Cold	2.35	0,28	88,09648663
3	Probase Cold	2.35	0,321	100,9963293
3	Probase Cold	2.35	0,308	96,90613529
3	Probase Cold	2.35	0,32	100,681699

3	Probase Cold	2.35	0,262	82,43314106
3	Probase Cold	0.94	0,27	84,95018353
3	Probase Cold	0.94	0,318	100,0524384
3	Probase Cold	0.94	0,284	89,35500787
3	Probase Cold	0.94	0,33	103,8280021
3	Probase Cold	0.94	0,297	93,44520189
4	Ufi Gel Hard	2.36	0,074	22,01289043
4	Ufi Gel Hard	2.36	0,091	27,0699058
4	Ufi Gel Hard	2.36	0,078	23,2027764
4	Ufi Gel Hard	2.36	0,074	22,01289043
4	Ufi Gel Hard	2.36	0,07	20,82300446
4	Ufi Gel Hard	2.36	0,073	21,71541894
4	Ufi Gel Hard	1.58	0,079	23,50024789
4	Ufi Gel Hard	1.58	0,091	27,0699058
4	Ufi Gel Hard	1.58	0,086	25,58254834
4	Ufi Gel Hard	1.58	0,07	20,82300446
4	Ufi Gel Hard	1.58	0,08	23,79771939
4	Ufi Gel Hard	1.58	0,086	25,58254834
4	Ufi Gel Hard	0.95	0,087	25,88001983
4	Ufi Gel Hard	0.95	0,093	27,66484879
4	Ufi Gel Hard	0.95	0,101	30,04462072
4	Ufi Gel Hard	0.95	0,095	28,25979177
4	Ufi Gel Hard	0.95	0,078	23,2027764
4	Ufi Gel Hard	0.95	0,084	24,98760535
4	Ufi Gel Hard	0.63	0,086	25,58254834
4	Ufi Gel Hard	0.63	0,101	30,04462072
4	Ufi Gel Hard	0.63	0,12	35,69657908
4	Ufi Gel Hard	0.63	0,103	30,63956371
4	Ufi Gel Hard	0.63	0,085	25,28507685
4	Ufi Gel Hard	0.63	0,085	25,28507685
5	Ufi Gel Hard	0.315	0,106	35,33333333
5	Ufi Gel Hard	0.315	0,113	37,66666667
5	Ufi Gel Hard	0.315	0,107	35,66666667
5	Ufi Gel Hard	0.315	0,128	42,66666667
5	Ufi Gel Hard	0.315	0,133	44,33333333
5	Ufi Gel Hard	0.315	0,116	38,66666667
5	Ufi Gel Hard	0.158	0,119	39,66666667
5	Ufi Gel Hard	0.158	0,165	55
5	Ufi Gel Hard	0.158	0,121	40,33333333
5	Ufi Gel Hard	0.158	0,119	39,66666667
5	Ufi Gel Hard	0.158	0,135	45
5	Ufi Gel Hard	0.158	0,124	41,33333333
5	Ufi Gel Hard	0.079	0,234	78

5	Ufi Gel Hard	0.079	0,302	100,6666667
5	Ufi Gel Hard	0.079	0,288	96
5	Ufi Gel Hard	0.079	0,285	95
5	Ufi Gel Hard	0.079	0,288	96
5	Ufi Gel Hard	0.079	0,301	100,3333333

7.4. IC50 determination tables

log(inhibitor) vs. normalized response -- Variable slope	MMA	Probase Cold
Best-fit values		
LogIC50	3,019	0,8527
HillSlope	-0,6571	-2,566
IC50	1044	7,124
Std. Error		
LogIC50	4,153	0,02334
HillSlope	1,210	0,3280
95% Confidence Intervals		
LogIC50	-5,348 to 11,39	0,8058 to 0,8997
HillSlope	-3,095 to 1,780	-3,226 to -1,906
IC50	4,483e-006 to 2,433e+011	6,394 to 7,937
Goodness of Fit		
Degrees of Freedom	46	49
R square	0,03525	0,9267
Absolute Sum of Squares	5823	4649
Sy.x	11,25	9,741
Number of points		
Analyzed	48	51

log(inhibitor) vs. normalized response -- Variable slope	HDMA	Ufi Gel Hard
Best-fit values		
LogIC50	-0,5663	-0,5873
HillSlope	-2,612	-0,8217
IC50	0,2715	0,2587
Std. Error		
LogIC50	0,01961	0,05422
HillSlope	0,2888	0,09657
95% Confidence Intervals		
LogIC50	-0,6056 to -0,5269	-0,6968 to -0,4777
HillSlope	-3,191 to -2,032	-1,017 to -0,6265
IC50	0,2480 to 0,2972	0,2010 to 0,3329
Goodness of Fit		
Degrees of Freedom	54	40
R square	0,9068	0,7339
Absolute Sum of Squares	7589	6284
Sy.x	11,85	12,53
Number of points		
Analyzed	56	42

log(inhibitor) vs. normalized response -- Variable slope	IBMA	Kooliner
Best-fit values		
LogIC50	0,5467	0,8126
HillSlope	-2,884	-5,942
IC50	3,521	6,496
Std. Error		
LogIC50	0,01758	0,007925
HillSlope	0,2309	0,5692
95% Confidence Intervals		
LogIC50	0,5115 to 0,5820	0,7966 to 0,8287
HillSlope	-3,347 to -2,421	-7,097 to -4,786
IC50	3,247 to 3,819	6,260 to 6,741
Goodness of Fit		
Degrees of Freedom	55	36
R square	0,9714	0,9799
Absolute Sum of Squares	2956	1250
Sy.x	7,331	5,893
Number of points		
Analyzed	57	38

log(inhibitor) vs. normalized response -- Variable slope	MA
Best-fit values	
LogIC50	1,504
HillSlope	-18,42
IC50	31,88
Std. Error	
LogIC50	0,006205
HillSlope	3,395
95% Confidence Intervals	
LogIC50	1,491 to 1,516
HillSlope	-25,27 to -11,57
IC50	30,97 to 32,81
Goodness of Fit	
Degrees of Freedom	43
R square	0,9291
Absolute Sum of Squares	5538
Sy.x	11,35
Number of points	
Analyzed	45